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Classification, Identification, and Clinical Significance of *Haemophilus* and *Aggregatibacter* Species with Host Specificity for Humans

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SUMMARY

The aim of this review is to provide a comprehensive update on the current classification and identification of *Haemophilus* and *Aggregatibacter* species with exclusive or predominant host specificity for humans. *Haemophilus influenzae* and some of the other *Haemophilus* species are commonly encountered in the clinical microbiology laboratory and demonstrate a wide range of patho-

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genicity, from life-threatening invasive disease to respiratory infections to a nonpathogenic, commensal lifestyle. New species of *Haemophilus* have been described (*Haemophilus pittmaniae* and *Haemophilus sputorum*), and the new genus *Aggregatibacter* was created to accommodate some former *Haemophilus* and *Actinobacillus* species (*Aggregatibacter aphrophilus*, *Aggregatibacter segnis*, and *Aggregatibacter actinomycetemcomitans*). *Aggregatibacter* species are now a dominant etiology of infective endocarditis caused by fastidious organisms (HACEK endocarditis), and *A. aphrophilus* has emerged as an important cause of brain abscesses. Correct identification of *Haemophilus* and *Aggregatibacter* species based on phenotypic characterization can be challenging. It has become clear that 15 to 20% of presumptive *H. influenzae* isolates from the respiratory tracts of healthy individuals do not belong to this species but represent nonhemolytic variants of *Haemophilus haemolyticus*. Due to the limited pathogenicity of *H. haemolyticus*, the proportion of misidentified strains may be lower in clinical samples, but even among invasive strains, a misidentification rate of 0.5 to 2% can be found. Several methods have been investigated for differentiation of *H. influenzae* from its less pathogenic relatives, but a simple method for reliable discrimination is not available. With the implementation of identification by matrix-assisted laser desorption ionization–time of flight mass spectrometry, the more rarely encountered species of *Haemophilus* and *Aggregatibacter* will increasingly be identified in clinical microbiology practice. However, identification of some strains will still be problematic, necessitating DNA sequencing of multiple housekeeping gene fragments or full-length 16S rRNA genes.

INTRODUCTION

Among the species of *Haemophilus* and *Aggregatibacter* addressed in this review, *Haemophilus influenzae* is clearly the most important human pathogen. The history of this bacterium is fascinating, as it has been involved in major medical and scientific achievements, sometimes incidentally. As indicated by its name, the bacterium has also been implicated in major misconceptions. When the world was suffering from pandemic influenza in 1889 and 1890, bacteriologists were vigorously pursuing the causative agent of the disease. The news of the discovery of the influenza bacillus by Richard Pfeiffer was a sensation, and a preliminary report was published simultaneously in January 1892 in German, English, and French medical journals (1). The investigation of the influenza bacillus was hampered by the difficulty of growing it on laboratory media. When it grew, it did so in minute, pinpoint-size colonies that could easily be overlooked or overgrown by other bacteria present in the sample. When Alexander Fleming discovered penicillin, he also observed the relative nonsusceptibility of the influenza bacillus. Penicillin-containing agars could therefore be used as selective media to increase the recovery of the microorganism, and Fleming promulgated this use in the title of his pioneering paper from 1929: "On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*" (2). A few years later, the discovery of the influenza virus rendered this particular use of penicillin less important (3). Seminal events in the chronicle of *H. influenzae* are listed in Table 1.

Louis Pasteur's work with the causative agent of fowl cholera, *Pasteurella multocida*, preceded Pfeiffer's discovery of *H. influenzae* (4). The bacterial family proposed in 1979 to accommodate the genera *Pasteurella*, *Actinobacillus*, and *Haemophilus* was consequently designated *Pasteurellaceae* (5). The family, which had expanded to 18 genera by 2012, encompasses strictly commensal organisms as well as opportunistic pathogenic species of considerable medical and veterinary importance. These bacteria colonize mucosal surfaces of humans and animals, and most species exhibit a strong association with specific hosts. Before the advent of molecular methods of identification, new species were allocated to the three classical genera based on relatively few, critical phenotypic markers. Species dependent on particular growth factors in blood belonged to the genus *Haemophilus*, while species without this dependence were classified with *Pasteurella* or *Actinobacillus*. By this definition, *Haemophilus* circumscribed bacterial species cultured from humans and various animals, and it emerged as a very heterogenous genus with the advent of molecular methods (6). Beginning with the transfer in 1983 of *Haemophilus pleuropnemoniae* to the genus *Actinobacillus*, as *Actinobacillus pleuropneumoniae* (7), six former *Haemophilus* species have now been classified with other genera within the family *Pasteurellaceae* (Table 2). Currently, there are four *Haemophilus* species with host specificity for animals: *Haemophilus felis*, *Haemophilus haemoglobinophilus*, *Haemophilus paracuniculus*, and *Haemophilus parasuis*. It is plausible that these will be reclassified in the future, and they are not dealt with further in this review.

The genus *Aggregatibacter* was created in 2006 to accommodate *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and *Haemophilus segnis*; these species were only distantly related to the type species of their former genera but were sufficiently related to each other to warrant creation of a new genus (8). Recently, *Aggregatibacter actinomycetemcomitans* was isolated from different Old World nonhuman primates (9), and the genome of a strain cultured from a rhesus macaque has been sequenced (10). Furthermore, 16S rRNA gene sequences with high similarity to *Aggregatibacter segnis* have been cloned from the canine oral microbiome (11). Thus, the human host specificity of the *Aggregatibacter* genus is not absolute.

Few species of *Pasteurellaceae* other than *Haemophilus* and *Aggregatibacter* species exhibit host specificity for humans. *Actinobacillus ureae* and *Actinobacillus hominis* are commensals of the oropharynx and upper respiratory tract (12) that occasionally cause infections in patients with underlying diseases. The most commonly

TABLE 1 Seminal events in the history of *Haemophilus influenzae*

Date	Event	Reference
1893	Pfeiffer publishes the discovery of the influenza bacillus	19
1917	Genus *Haemophilus* is created, with type species *Haemophilus influenzae*	297
1921	Thjötta and Avery differentiate two separate growth factors present in blood	22
1929	Alexander Fleming describes penicillin as a means of isolating *H. influenzae*	2
1931	Margaret Pittman describes capsulation and the association of type b with meningitis	50
1935	Influenza virus is discovered	3
1980s	*H. influenzae* serotype b vaccination is implemented in many parts of the world	
1995	*H. influenzae* becomes the first free-living organism to have its genome sequenced	41

TABLE 2 Former species of *Haemophilus* transferred to other genera

Former name	Event	Reference
H. aphrophilus	Transferred to genus *Aggregatibacter*	8
H. avium	Transferred to genus *Avibacterium*	33, 298
H. paragallinarum	Transferred to genus *Avibacterium*	33
H. paraphrophilus	Later heterotypic synonym of *H. aphrophilus* (transferred to genus *Aggregatibacter*)	8
H. pleuropneumoniae	Transferred to genus *Actinobacillus*	7
H. segnis	Transferred to genus *Aggregatibacter*	8

reported infection with *A. ureae* is meningitis (13), while *A. hominis* is seen mainly as the cause of pulmonary infections (14). *Pasteurella bettyae* is a commensal of the genitourinary tract and has been isolated from human Bartholin gland abscesses, urine, and finger infections (15, 16). The ecology and significance of these bacteria have received little attention (12), and infections are probably underreported (14). *P. multocida* shows host specificity for various domesticated animals but can cause severe human infections after introduction into wounds by bites or licks (17, 18). Phenotypic characteristics of *A. ureae*, *A. hominis*, *P. bettyae*, and *P. multocida* are included in Table 3, but otherwise these species are not considered further in this review. Figure 1 shows a DNA sequence-based phylogenetic comparison of the nine *Haemophilus* and three *Aggregatibacter* species that are covered in the present review.

In addition to descriptions of new species and a new genus, the merger and renaming of other species in the *Haemophilus* and *Aggregatibacter* genera have resulted in the obsolescence of previously familiar names. Fortunately, certain issues have become simpler, such as the crystallization of the genus *Aggregatibacter* as a group of bacteria associated predominantly with humans. A reorganization of the genus *Haemophilus* may ultimately end with a similar delineation. DNA sequencing is increasingly used for iden-

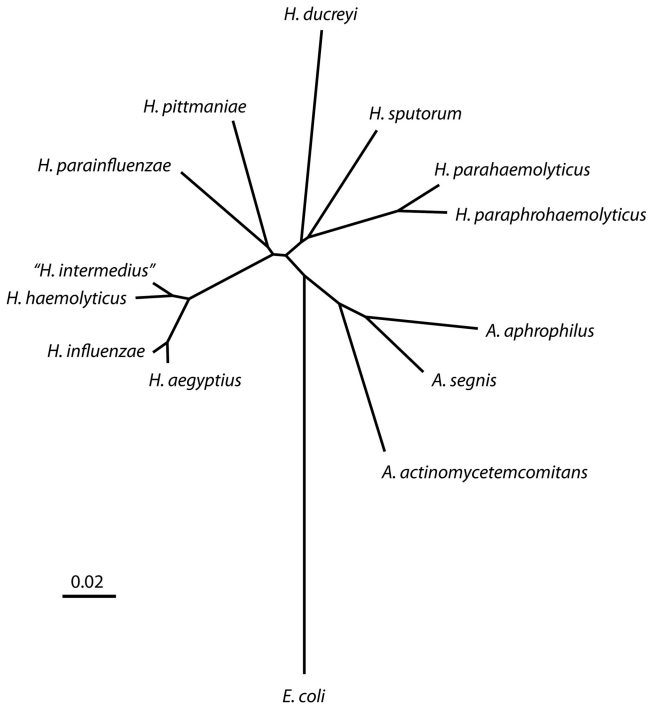

FIG 1 Genetic relationships of *Haemophilus* and *Aggregatibacter* species, using *Escherichia coli* as an outgroup. Concatenated sequences of near-full-length 16S rRNA genes (1,361 to 1,364 nt) plus fragments of three housekeeping genes, *infB*, *pgi*, and *recA* (1,293 nt), were compared by the neighbor-joining method (36, 99). The dendrogram is based on type strains of validated species, except for *H. ducreyi*, where strain 35000HP was used (GenBank accession no. AE017143). Strain CCUG 11096 represents the not validly named species *H. intermedius*. Bar, 2 substitutions per 100 nucleotides.

TABLE 3 Selected phenotypic characters for differentiation of *Pasteurellaceae* species isolated from humans[a]

	Phenotype															
	Haemophilus sp.									*Aggregatibacter* sp.			*Actinobacillus* sp.		*Pasteurella* sp.	
Character	infl	aegy	haem	pinf	phae	pphae	sput	pitt	ducr	acti	aphr	segn	homi	ureae	bett	mult
Porphyrin synthesis (X factor not required)	0	0	0	+	+	+	+	+	0	+	+	+	+	+	+	+
NadV synthesis (V factor not required)	0	0	0	0	0	0	0	0	+	+	d	0	+	+	+	+
Catalase	+	+	+	d	d	d	d	d	0	+	0	d	+	d	0	+
Hemolysis	0	0	+	d	+	+	+	+	d	0[b]	0	0	0	0	0	0
β-Galactosidase	0	0	0	d	0	+	+	+	0	0	+	d	+	0	0	0
Tryptophanase	d	0	d	d	0	0	0	0	0	0	0	0	0	0	+	+
Urease	d	+	+	d	+	+	+	0	0	0	0	0	+	+	0	0
ODC	d	0	0	d	0	0	0	0	0	0	0	0	0	0	0	+
Acid from:																
Sucrose	0	0	0	+	+	+	+	+	0	0	+	w	+	+	0	+
Mannose	0	0	0	+	0	0	0	+	0	d	+	w	d	d	d	+
Lactose	0	0	0	0	0	0	0	0	0	0	+	0	+	0	0	0
IgA1 protease	+	+	0	0	+	0	0	0	0	0	0	0	0	0	0	0

[a] Interpretations: +, positive; 0, negative; d, variable; w, weak or delayed reaction. Abbreviations: infl, *H. influenzae*; aegy, *H. aegyptius*; haem, *H. haemolyticus*; pinf, *H. parainfluenzae*; phae, *H. parahaemolyticus*; pphae, *H. paraphrohaemolyticus*; sput, *H. sputorum*; pitt, *H. pittmaniae*; ducr, *H. ducreyi*; acti, *A. actinomycetemcomitans*; aphr, *A. aphrophilus*; segn, *A. segnis*; homi, *A. hominis*; bett, *P. bettyae*; mult, *P. multocida*; ODC, ornithine decarboxylase; IgA1, immunoglobulin A1.
[b] Isolates with overexpression of leukotoxin may exhibit a zone of hemolysis (272).

tification and typing, and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry holds promise for revolutionizing routine identification in the clinical microbiology laboratory.

The aim of this review is to provide a comprehensive update on current classification and identification methods for *Haemophilus* and *Aggregatibacter* species. Particular emphasis is put on the difficult differentiation of *H. influenzae* from *Haemophilus haemolyticus* and related organisms. The clinical significance of *Haemophilus* and *Aggregatibacter* is reviewed briefly, with a focus on the consequences of recent taxonomic rearrangements, in addition to an update on the association of particular species with various clinical syndromes. A number of pertinent reviews are listed for more in-depth information on general aspects of the clinical significance of these organisms.

GROWTH FACTOR DEPENDENCE OF *HAEMOPHILUS* AND *AGGREGATIBACTER*

Two defective metabolic pathways result in dependence on specific growth factors that are traditionally referred to as X (heme) and V (NAD), and this dependence has played a major role in the etymology and delineation of *Haemophilus* (Gr. *haima*, blood; Gr. *philus*, friend, lover; *Haemophilus*, the blood lover). Richard Pfeiffer succeeded in culturing the "influenza bacillus" by inclusion of blood in the growth medium and, furthermore, showed that hemoglobin was the essential constituent of the blood (19). A few years later, Grassberger confirmed the necessity of hemoglobin for propagation of the influenza bacillus, but he also noticed the luxurious growth around colonies of other bacteria plated on the medium (20). The latter accessory factor could be supplied by plant or animal tissue and was destroyed by autoclaving (21). In 1921, Thjötta and Avery finally coined the terms V factor, for the vitamin-like, heat-labile substance, and X factor, for the less-defined, heat-stable substance associated with hemoglobin and acting in minute amounts (22). Although these specific growth factors are no longer decisive taxonomic criteria, they continue to be phenotypic traits of great practical importance.

X Factor and Biosynthesis of Heme

The heme biosynthetic pathway is common to animals, plants, and bacteria, irrespective of whether the final end product is cytochrome, hemoglobin, or chlorophyll, and lack of the ability to synthesize heme is rare in biology (23). The formation of protoporphyrin begins with the condensation of two linear δ-aminolevulinic acid molecules into the five-membered pyrrole ring, porphobilinogen. Four porphobilinogen molecules condense and circularize into uroporphyrinogen III, which is modified by side chain substitutions in successive enzymatic steps. Finally, protoheme is formed from protoporphyrin by chelation of ferrous iron. The genome of *H. influenzae* encodes ferrochelatase (*hemH*), and protoporphyrin is the minimal biochemical equivalent of X factor (24, 25); occasional strains fail to synthesize protoheme from protoporphyrin, and then protoheme is the minimal equivalent of X factor (26, 27). For many years, the main obstacles to the development of a satisfactory classification and identification scheme for *Haemophilus* were methodological problems with identification of the X factor requirement (28).

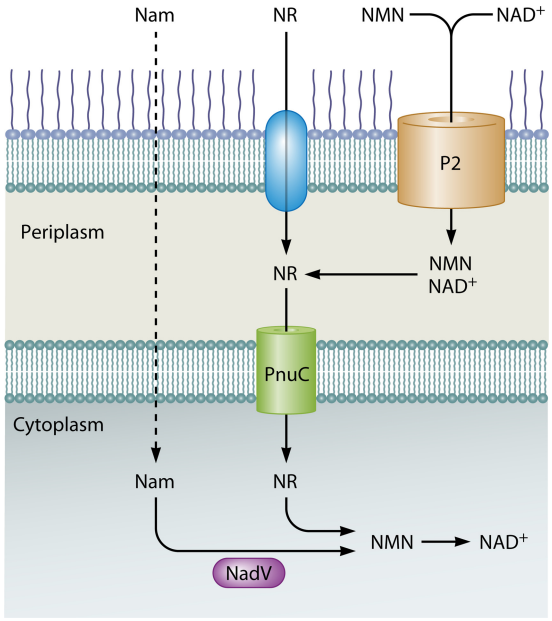

FIG 2 NAD utilization in *Pasteurellaceae*. Nicotinamide mononucleotide (NMN) and NAD enter the periplasm through the general porin OMP P2 and are degraded to nicotinamide riboside (NR); exogenous NR probably enters the periplasm through a different porin (293). NR is internalized through a cytosolic membrane-located permease (PnuC) and serves as the substrate for a resynthesizing enzyme which uses ATP to generate NAD. Nicotinamide (Nam) freely diffuses through the cell membranes and can serve as the substrate for those members of the family that express a functional nicotinamide phosphoribosyltransferase (NadV). (Based on reference 30.)

V Factor and Biosynthesis of NAD

Studies in *Enterobacteriaceae* have shown that more than 20 proteins are involved in the biosynthesis, recycling, and uptake of NAD. NAD is synthesized by a *de novo* pathway and by the pyridine salvage pathway, which recycles degradative products of NAD back to NAD (29). In contrast, the reduced set of processes in *Pasteurellaceae* is restricted to uptake of NAD (30), and all *Pasteurellaceae* organisms acquire this essential nutrient from their environment, either as NAD or as a limited number of NAD precursors. However, some species of *Pasteurellaceae* are capable of utilizing nicotinamide in a reaction catalyzed by the enzyme nicotinamide phosphoribosyltransferase (NadV) (Fig. 2). As ample nicotinamide is present in complex media, such isolates do not show a dependence on V factor in the laboratory. Growth media without nicotinamide can be prepared, and on such media, all *Pasteurellaceae* organisms will exhibit dependence on V factor (31).

The current definition of V factor dependence is therefore synonymous with the absence of the enzyme NadV. With *Aggregatibacter aphrophilus*, some isolates are dependent on V factor (formerly *Haemophilus paraphrophilus*), while others are not (formerly *Haemophilus aphrophilus*). V-factor-dependent strains carry a *nadV* pseudogene, and in strains competent for transformation, the V-factor-dependent phenotype is naturally reversible by transfer of the intact *nadV* gene (8). V-factor-dependent and -independent biovars have also been documented for *Actinobacillus pleuropneumoniae* (7), *Haemophilus parainfluenzae* (32), and *Avibacterium paragallinarum* (33, 34). Dependence on V factor is therefore not a crucial character for identification and classifica-

tion of *Pasteurellaceae* species, as occasional strains may give aberrant results. Nevertheless, V factor dependence is a highly valuable test in the initial characterization of presumptive members of *Pasteurellaceae*.

THE GENUS *HAEMOPHILUS*

Nine validly described species of the genus *Haemophilus* demonstrate host specificity for humans. They may be divided into three groups that share certain phenotypic traits: the *H. influenzae* group, consisting of the three X-factor-dependent species, i.e., *H. influenzae*, *Haemophilus aegyptius*, and *H. haemolyticus*; the *H. parainfluenzae* group, consisting of the five X-factor-independent species, i.e., *H. parainfluenzae*, *Haemophilus parahaemolyticus*, *Haemophilus paraphrohaemolyticus*, *Haemophilus pittmaniae*, and *Haemophilus sputorum*; and a group encompassing only *Haemophilus ducreyi*. Phenotypic characteristics differentiating the nine described species of *Haemophilus* from other *Pasteurellaceae* species are shown in Table 3.

The *Haemophilus influenzae* Group

The salient characteristic of the *Haemophilus influenzae* group is a deficient heme biosynthetic pathway resulting in X-factor-dependent growth *in vitro*. Occasional heme-synthesizing strains have been documented ("*Haemophilus intermedius* subsp. *intermedius*") (35); it has been suggested that such strains may represent an ancestral genotype from which X-factor-dependent *H. influenzae* evolved (36). Three named species belong to the group, namely, *H. influenzae*, *H. aegyptius*, and *H. haemolyticus*, together with a broad range of unnamed taxa with various phenotypic and genotypic traits. All species and unnamed taxa in this group, including X-factor-independent representatives of "*Haemophilus intermedius*," are negative for β-galactosidase. *H. influenzae* is the type species of the genus *Haemophilus*, and the *H. influenzae* group is sometimes referred to as "genus *Haemophilus sensu stricto*" (37, 38).

Haemophilus influenzae. Isolates of *H. influenzae* transport and metabolize carbohydrates through the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS), but unlike the PTS found in enteric bacteria, the PTS in *H. influenzae* is specific for fructose (39). In the absence or at a reduced level of fructose, transport and metabolism of fucose and other sugars are upregulated (40). The genome sequence of *H. influenzae* has revealed the presence of a cluster of genes involved in the transport and subsequent metabolism of fucose (41, 42). One of the genes in the fucose operon is *fucK*, encoding fuculokinase, which has been included in the multilocus sequence typing (MLST) scheme for *H. influenzae* (43). The presence of *fucK* is specific for *H. influenzae* and has been used to identify *H. influenzae* and to differentiate it from *H. haemolyticus* and related organisms (see below).

H. influenzae demonstrates a heterogenous phenotype and is separated into eight biotypes based on the variable characters tryptophanase (indole production), urease, and ornithine decarboxylase (ODC) (Table 4). More than 90% of isolates produce urease (28, 44). The urease gene cluster is among the most highly upregulated genes in the chinchilla animal model of otitis media (45) and in cultures in pooled human sputum (46), and urease activity enhances survival of *H. influenzae* at a reduced pH (47). If urease expression has importance for survival and replication in the human respiratory tract, it may account for the high prevalence of this phenotypic trait. While fermentation of xylose, ribose, and galactose is a well-known metabolic characteristic of *H. influenzae*, the differentiating property of fucose fermentation remains to be addressed.

TABLE 4 Biotypes of *Haemophilus influenzae* and *Haemophilus parainfluenzae*[a]

	Phenotype															
	H. influenzae biotypes								*H. parainfluenzae* biotypes							
Character	I	II	III	IV	V	VI	VII	VIII	I	II	III	IV	V	VI	VII	VIII
Indole	+	+	0	0	+	0	+	0	0	0	0	+	0	+	+	+
Urease	+	+	+	+	0	0	0	0	0	+	+	+	0	0	+	0
ODC	+	0	0	+	+	+	0	0	+	+	0	+	0	+	0	0

[a] As defined by Kilian (48).

(i) Capsulation. Strains of *H. influenzae* may produce one of six distinct capsular polysaccharides or may be unencapsulated. The capsules consist of repeating units of one of six different disaccharides (48, 49). The presence of polysaccharide capsular antigen, originally described by Margaret Pittman in 1931 (50), provides the basis for serotype designations a to f (Hia to Hif). Unencapsulated *H. influenzae* strains are commonly referred to as nontypeable *H. influenzae* (NTHI). The genetic capsulation locus is composed of three functionally distinct regions (51, 52). Regions I and III are common to all six capsular types and contain genes involved in the export and processing of the capsular material. Region I genes (*bexDCBA*) code for an ATP-driven capsule export apparatus (53), while region III genes (*hcsAB*) are necessary for transport of polysaccharide across the outer membrane (54). Region II carries serotype-specific biosynthesis genes unique to each of the six capsule types; the regions from representatives of each serotype have been sequenced and comprise three to eight genes (49, 55–58). Assignment to serotypes may be done by slide agglutination or by PCR; however, slide agglutination carries a high rate of discordance compared with PCR-based methods (59–63).

(ii) Population structure. The first insight into the population structure of *H. influenzae* was obtained using multilocus enzyme electrophoresis (MLEE). These studies revealed that capsulated *H. influenzae* populations were highly clonal (64) and could be divided into two divisions (I and II) (65). Unencapsulated isolates were more diverse than encapsulated isolates, and their population structure appeared to be more influenced by recombination (66). However, unlike the case for other naturally competent bacteria, such as pneumococci or meningococci, the amount of homologous recombination in *H. influenzae* did not blur phylogenetic signals (67). An MLST scheme for *H. influenzae* was subsequently established (43). Concatenation of MLST sequences is the basis of phylogenetic comparison of multiple housekeeping gene fragments by multilocus sequence analysis (MLSA) (68). By MLSA, the bipartite division of capsulated strains was confirmed, but in contrast to the population structure revealed by MLEE, strains of serotype e did not cluster with division I strains. Consequently, the designations of phylogenetic groups I and II were introduced (43), with group I encompassing the core of the species, including the type strain, all strains of serotypes c and d, the majority of strains of serotypes a and b, and most unencapsulated isolates, and the smaller phylogenetic group II encompassing serotypes e and f, some strains of serotypes a and b, and some unencapsulated isolates. With the increasing number of sequence

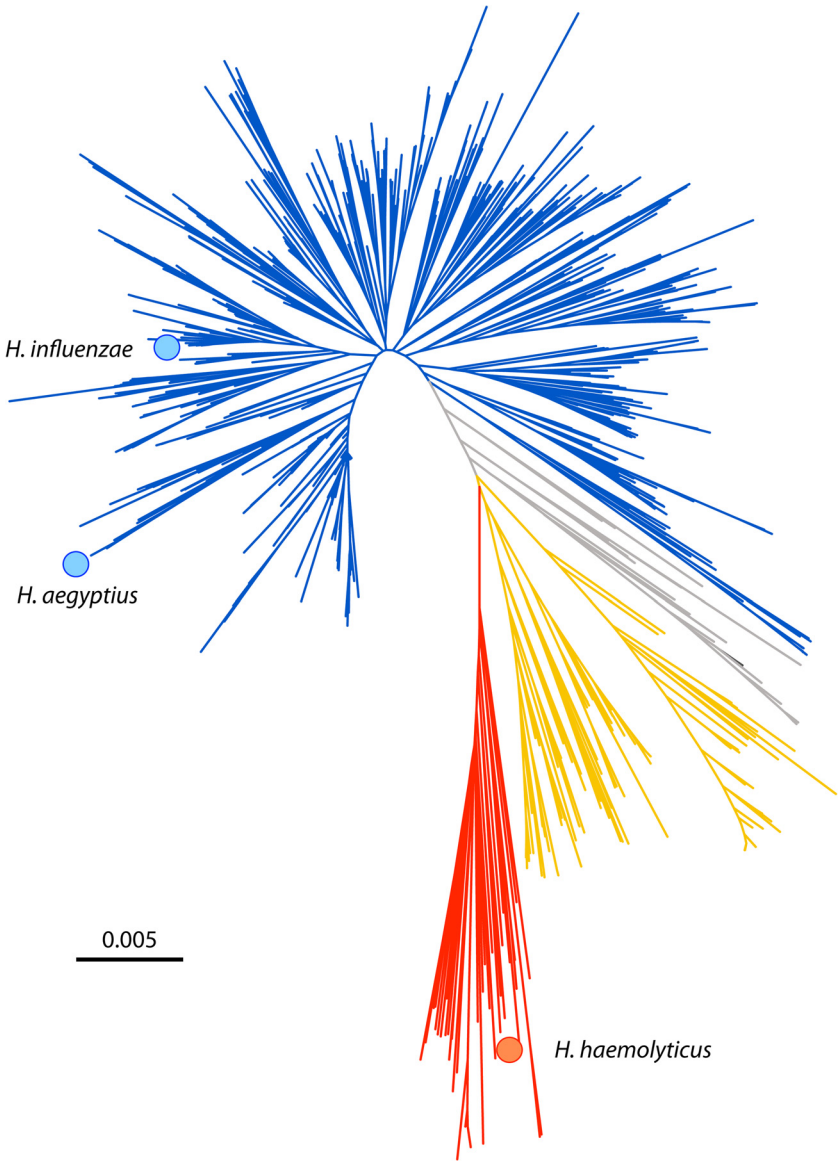

FIG 3 Neighbor-joining dendrogram based on concatenated gene fragments of *adk*, *atpG*, *frdB*, *mdh*, *pgi*, and *recA* (2,712 nucleotides), comparing the type strains of *H. influenzae*, *H. aegyptius*, and *H. haemolyticus* (filled circles), five genome-sequenced *H. haemolyticus* strains (294), and 30 strains of *H. haemolyticus* and related organisms (36), with 900 *H. influenzae* sequence types downloaded from the MLST website (www.mlst.net). The 36 strains of *H. haemolyticus* and related organisms are negative for *fucK*, and gaps were treated by complete deletion using MEGA, version 5 (295). Phylogenetic group I is indicated in blue, phylogenetic group II in yellow, and *H. haemolyticus* and related organisms in red. Strains with equivocal allocation to phylogenetic groups are shown in gray. Bar, 1 substitution per 200 nucleotides.

types (STs) being deposited in the MLST database, it has become difficult to make a clear distinction between phylogenetic groups I and II for unencapsulated isolates (Fig. 3; the figure is restricted to six genes). For encapsulated strains, the division into phylogenetic groups is supported by other means. The capsule loci are associated with either the insertion element IS*1016* (group I) or the *sodC* gene (group II) (57), and comparison of *bexA* gene sequences from 36 encapsulated isolates also resulted in two clusters in accordance with the phylogenetic groups (69).

A somewhat different population structure was suggested based on maximum parsimony analysis of 359 MLST sequence types, using sequence type 65 of the major phylogenetic group I as the outgroup (70). The study identified 13 clades containing 6 to 89 STs, while 80 STs were not included in any of the clades. Clade 2 corresponds closely to phylogenetic group II, and the major modifications are the dissection of phylogenetic group I into the remaining 12 clades and STs outside clades. Most recently, a statistical (Bayesian) genetic analysis of 819 distinct *H. influenzae* MLST genotypes was performed (67). The clusters obtained by Bayesian analysis correlate well with the classical subdivisions of the *H. influenzae* population but show limited concordance with the clades of Erwin et al. (70). Compared with encapsulated isolates, a significantly larger proportion of unencapsulated isolates showed evidence of recombination, and when admixture was present, the total amount of recombination per strain was greater for unencapsulated strains (67). Capsulation by itself was not a

TABLE 5 Mass spectrometry log score similarities of test strains to type strains of species, calculated with Biotyper 3 software (Bruker Daltronic)[a]

Test strain	Species	Similarity (log score) to type strain												
		1	2	3	4[b]	5	6	7	8	9	10	11	12	13
1	*H. influenzae*	**2.09**	2.02	1.71	1.77									
2	*H. aegyptius*[c]	2.29	**2.33**	1.87	1.96	1.54			1.57					
3	*H. haemolyticus*	1.90	1.82	**2.21**	2.13	1.45			1.43					
4	Cryptic genospecies[d]	1.63	1.67	2.07	**2.12**	1.45			1.53					
5	*H. parainfluenzae*					**2.40**			1.61					
6	*H. parahaemolyticus*						**2.18**	2.00	1.46					
7	*H. paraphrohaemolyticus*[e]						1.82	**2.38**		1.57				
8	*H. pittmaniae*				1.42	1.77	1.43		**2.40**	1.41				
9	*H. sputorum*									**2.26**				
10	*H. ducreyi*[f]										**1.80**			
11	*A. actinomycetemcomitans*											**2.07**		
12	*A. aphrophilus*												**2.22**	
13	*A. segnis*								1.41			1.55		**2.17**

[a] Two or three test strains from each species were selected to cover the genetic diversity of the species (8, 36, 98, 99, 101) and compared with reference spectra of the type strains generated by the direct-smear method (99). Results are expressed as logarithmic means for measurements of test strains performed in triplicate; only log scores of >1.4 are shown. Results in bold are similarity scores of test strains compared to the type strain of the same species.
[b] Cryptic genospecies biotype IV strain S32F2 (94) was used as a reference.
[c] Original strains from cases of acute conjunctivitis, designated *H. aegyptius* by Margaret Pittman (strains 178a, 758, and 763) (71, 78).
[d] *Haemophilus intermedius* subsp. *intermedius* (CCUG 11096), *H. intermedius* subsp. *gazogenes* (CCUG 15793), and nonhemolytic *H. haemolyticus* (HK 855) (36).
[e] Two isolates compatible with *H. paraphrohaemolyticus* by phenotype and DNA sequence were available (99).
[f] Two isolates were included (CCUG 39065 and 35000HP).

barrier to transformation, and factors other than the capsule may constitute decisive determinants of the recombination rate in the *H. influenzae* population.

***Haemophilus aegyptius* and *H. influenzae* biogroup aegyptius.** *H. aegyptius* was described by Pittman and Davis in 1950, as a species distinct from *H. influenzae* and with a particular propensity to cause conjunctivitis (71). It was believed to be the bacterium that Robert Koch noticed by microscopic examinations of purulent matter from cases of eye inflammation in Egypt in 1883 and that later was propagated *in vitro* by the American ophthalmologist John Weeks (the Koch-Weeks bacillus). The description by Pittman and Davis was based on a field investigation of 28 strains from cases of acute conjunctivitis in the lower Rio Grande Valley of Texas. The authors stated that *H. aegyptius* could be separated from *H. influenzae* by "serological means and, to a certain extent, by growth characteristics and biochemical reactions" (71). Pertinent phenotypic traits of *H. aegyptius* were the inability to ferment xylose or produce indole and the ability to agglutinate human erythrocytes.

A controversy has existed for years on whether *H. aegyptius* should be classified separately from *H. influenzae*. Some investigators have differentiated the two species by differences in the ability to grow on tryptic soy agar, troleandomycin susceptibility, cell morphology, and outer membrane protein (OMP) profile (72, 73), but all of these tests have subsequently been disputed or discredited (73–75). DNA-based methods show that *H. aegyptius* and *H. influenzae* do not merit separate species rank: the type strain of *H. aegyptius* cannot be separated from *H. influenzae* by DNA hybridization (74), and it is located within the core of the species by MLSA (Fig. 3). Steps to formally combine the two species have not been taken and are complicated by the fact that the specific epithet "*aegyptius*" has priority over "*influenzae*" (1). A pragmatic solution is to accept *H. aegyptius* as a validly named species that designates a group of strains related to *H. influenzae* isolated during a short period from a single geographic region and to refrain from wider use of the name. The type strain plus three other original Pittman strains were used for the MALDI-TOF mass spectrometry measurements presented in Table 5, and this analysis also testifies to the close relationship of the two species.

Brazilian purpuric fever (BPF) appeared in small outbreaks in Brazil in the 1980s as a syndrome characterized by epidemic purpura fulminans preceded by purulent conjunctivitis (76, 77). The disease was caused by a single clone of *Haemophilus*, the BPF clone, which had the characteristics of *H. aegyptius*. Measurement of DNA relatedness by hybridization clearly indicated that the BPF clone and reference strains of *H. aegyptius* and *H. influenzae* all belonged to the same species; in consequence, Brenner and coworkers introduced the informal designation *H. influenzae* biogroup aegyptius for strains of *H. aegyptius*, including the BPF clone (76). As stated above, there are formal obstacles to the unification of the two species, and the causative agent of Brazilian purpuric fever is more correctly referred to as the BPF clone of *H. influenzae*. Representatives of the BPF clone were compared with the original Pittman strains of *H. aegyptius* and other *Haemophilus* strains by MLEE (78). By this method, the BPF clone was related to isolates from cases of conjunctivitis from Brazil and Texas, and all of these strains were characterized by rod-shaped morphology, microcolony formation on conjunctival cells, and a 40-bp frameshift deletion in the *Haemophilus* adhesion and penetration gene *hap* (78). However, the close relationship of the type strain of *H. aegyptius* with the BPF clone reference strain F3031 was not substantiated by comprehensive genome hybridization data (79) or MLSA (70). Genome sequencing of the BPF clone of *H. influenzae* and a contemporaneous, non-BPF-associated conjunctivitis strain from Brazil recently showed that the two strains are characterized by a number of novel adhesins, including a 10-member family of trimeric autotransporter adhesins, unique high-molecular-weight proteins, and four novel fimbrial operons (80). Thus, the tropism for the eye may be related to a particular repertoire of adhesins expressed by the original Pittman strains of *H. aegyptius*

as well as several lineages of *H. influenzae*, including the BPF clone and non-BPF-associated conjunctivitis strains.

Haemophilus haemolyticus. The specific name *H. haemolyticus* was introduced in the first edition of *Bergey's Manual of Determinative Bacteriology* in 1923 (81). With the publication of the name *Haemophilus parahaemolyticus* in 1953 for X-factor-independent strains (82), *H. haemolyticus* was limited to hemolytic *Haemophilus* strains dependent on both the X and V factors. Such strains have been considered rare and of little clinical significance, and only a few isolates of this species were included in the influential phenotypic study of the *Haemophilus* genus by Kilian (28), with a reported phenotype as presented in Table 3. However, recent studies have shown that 12 to 40% of X- and V-factor-dependent strains from the respiratory tract do not belong to *H. influenzae* (83–88), and it has been proposed that such strains be classified as *H. haemolyticus* (37, 86). Many of the strains thus excluded from *H. influenzae* do not comply by phenotype with the classical description of *H. haemolyticus*: a large fraction of strains are nonhemolytic (85–87), and variable results in tests for urease and ornithine decarboxylase allocate strains to seven different biotypes (37) rather than the two classical biotypes (II and III) based on tryptophanase production (48) (Tables 3 and 4). The diversity of strains of the species *H. haemolyticus* may be real and merely brought to light by the renewed interest in the species. This would mirror the increase in the number of biotypes of *H. influenzae*, as only five biotypes were introduced in the original description by Kilian, which was based on 185 strains (28).

The difficult delineation of *H. haemolyticus* is accentuated by the existence of other, genetically related taxa with different phenotypes, ecological niches, or pathogenicities: the so-called cryptic genospecies biotype IV and *Haemophilus intermedius* (see below). A number of such strains formed a coherent sequence cluster with genuine *H. haemolyticus* strains as evaluated by a six-gene MLSA (36). To classify all variant strains and cryptic genospecies as *H. haemolyticus* would be convenient, because *H. haemolyticus* is a validated specific epithet in proximity to, but distinct from, *H. influenzae*. By MALDI-TOF mass spectrometry analysis, only small differences are observed between reference strains of *H. haemolyticus*, "cryptic genospecies biotype IV," and *H. intermedius* (Table 5), which indicates that their unification in a single species would be operational if routine identification was based on mass spectrometry. However, an emended description of *H. haemolyticus* has not been formally suggested. In the present review, the designation *H. haemolyticus* is reserved for isolates that conform to the classical phenotype, i.e., hemolytic X- and V-factor-dependent *Haemophilus* isolates positive for urease and negative for ornithine decarboxylase.

(i) Cryptic genospecies biotype IV. Unusual *Haemophilus* strains isolated from the genitourinary tract were first reported from Canada (89, 90) and have been studied further by Quentin and coworkers (91, 92); they are sometimes referred to as "*Haemophilus quentini*" (93, 94). Such strains have the phenotypic characteristics of *H. influenzae* biotype IV (negative for tryptophanase/indole production and positive for urease and ornithine decarboxylase) but can be differentiated from *H. influenzae* by sequencing of 16S rRNA or housekeeping genes, and thus they represent a cryptic genospecies (36, 92, 94). An active copper-zinc-cofactored superoxide dismutase (CuZnSOD) has been described as a phenotypic means to discriminate cryptic genospecies biotype IV from biotype IV strains of *H. influenzae* (95). By MLSA, strains of cryptic genospecies biotype IV cluster with *H. haemolyticus* and related organisms excluded from *H. influenzae* (36). MALDI-TOF mass spectrometry also reveals a high similarity of cryptic genospecies biotype IV with *H. haemolyticus* (Table 5).

(ii) *Haemophilus intermedius.* Based on DNA hybridization and selected phenotypic traits, the species *Haemophilus intermedius* was proposed in 1989 (35), but the species is not validly named and has no standing in nomenclature. Two subspecies were described: *Haemophilus intermedius* subsp. *intermedius*, which was capable of synthesizing porphyrin from δ-aminolevulinic acid and fermenting sucrose, and *Haemophilus intermedius* subsp. *gazogenes*, which was capable of fermenting mannose and producing gas from glucose. Only fermentation of mannose could differentiate the latter subspecies from "nonhemolytic *H. haemolyticus*." A number of strains with the characteristics of *Haemophilus intermedius* were examined by 16S rRNA and housekeeping gene sequencing (36). The synthesis of porphyrins and independence of X factor were confirmed for *Haemophilus intermedius* subsp. *intermedius*. Several heme biosynthesis genes were identified and found to be carried chromosomally and flanked by the same genes as those observed with other members of the *Pasteurellaceae*. By a six-gene MLSA, these strains were related to *H. haemolyticus* and other taxa excluded from *H. influenzae*. Biosynthesis of heme in strains closely related to *H. haemolyticus* and *H. influenzae* has thus been documented, and this challenges long-held delineations in the genus *Haemophilus*. Porphyrin-synthesizing strains of *H. intermedius* will typically be misidentified as *H. parainfluenzae* by abbreviated phenotypic testing; negative results for β-galactosidase, maltose, and mannose are not typical for *H. parainfluenzae* and should raise the suspicion of a variant strain. MALDI-TOF mass spectrometry analysis reveals the high similarity of such strains with *H. haemolyticus* and related organisms (Table 5).

The *Haemophilus parainfluenzae* Group

The salient characteristic of the *Haemophilus parainfluenzae* group is the ability to synthesize heme, permitting growth *in vitro* in the absence of exogenously added X factor. Five named species belong to this group: *H. parainfluenzae*, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. pittmaniae*, and *H. sputorum*. All species ferment sucrose. Although most isolates of *H. parainfluenzae* are nonhemolytic, hemolysis is a distinct feature of the other species in the group.

H. parainfluenzae. Shortly after the description of the X and V factors, bacteria with a close resemblance to *H. influenzae* but dependent only on V factor were identified (96). The epithet "*parainfluenzae*" was devised for these strains, inaugurating a long tradition within the genus *Haemophilus* of the use of the prefix "*para*-" to highlight that such species resemble existing species but differ in growth factor requirements. A heterogenous phenotype is characteristic of *H. parainfluenzae* (Table 3). Similar to *H. influenzae*, strains of *H. parainfluenzae* can be separated into eight biotypes based on the variable characters tryptophanase, urease, and ornithine decarboxylase (Table 4). The biotype numbers do not designate similar test patterns for *H. influenzae* and *H. parainfluenzae*, because biotypes were originally numbered according to the prevalences of the phenotypic traits in the two species (28). The biotypes of *H. influenzae* and *H. parainfluenzae* can be determined by commercial test systems (97). Phenotypic discrimination of *H. parainfluenzae* from other *Haemophilus* and *Aggregati-*

bacter species is usually unproblematic. However, extended phenotypic characterization may be needed to discriminate *H. parainfluenzae* biotype III from *H. parahaemolyticus*, *H. paraphrohaemolyticus*, or *H. sputorum* and *H. parainfluenzae* biotype V from *H. pittmaniae* or V-factor-dependent strains of *Aggregatibacter* (Table 3) (8, 98, 99). *H. parainfluenzae* is distinct from other *Haemophilus* and *Aggregatibacter* species by 16S rRNA and housekeeping gene sequences (6, 98, 100) (Fig. 1). Delineation of *H. parainfluenzae* by MALDI-TOF mass spectrometry appears to be robust (Table 5).

Hemolytic species. Four species of the *H. parainfluenzae* group are hemolytic, namely, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. pittmaniae*, and *H. sputorum*. *H. pittmaniae* is negative for production of tryptophanase, urease, and ornithine decarboxylase, while *H. parahaemolyticus*, *H. paraphrohaemolyticus*, and *H. sputorum* are positive for urease. Strains of *H. parainfluenzae* may express hemolysin, but hemolytic strains of this species are usually positive for both urease and ornithine decaboxylase (48, 101).

H. paraphrohaemolyticus was originally described as a species distinct from *H. parahaemolyticus* due to its CO_2-stimulated growth (102), but this phenotypic trait is unreliable; indeed, one of the three original strains deposited by Zinnemann and coworkers was later shown to belong to *H. parainfluenzae* (99). Instead, *H. parahaemolyticus* has been separated from *H. paraphrohaemolyticus* by the former's ability to produce IgA1 protease and the latter's ability to produce β-galactosidase (48). It was recently shown that the majority of isolates with a phenotype consistent with *H. paraphrohaemolyticus*, i.e., similar to *H. parahaemolyticus* but positive for β-galactosidase and negative for IgA1 protease, did not belong to *H. paraphrohaemolyticus* but constituted a separate taxon designated *H. sputorum* (99). *H. parahaemolyticus* is closely related to *H. paraphrohaemolyticus* by both 16S rRNA gene comparison and a three-gene MLSA, and the two species cannot be differentiated by current MALDI-TOF mass spectrometry analysis (99). The rare occurrence of *H. paraphrohaemolyticus* hinders a thorough characterization of the species, which is a prerequisite for certain delineation from *H. parahaemolyticus*. At present, the designation *H. paraphrohaemolyticus* should be restricted to rare isolates that are positive for β-galactosidase and for which DNA sequencing or mass spectrometry has indicated identification as *H. parahaemolyticus*.

While *H. parahaemolyticus* and *H. paraphrohaemolyticus* are closely related by DNA sequence and mass spectrometry patterns, *H. sputorum* and *H. pittmaniae* form distinct and separate lineages (Fig. 1 and Table 5). The genome sequence of *H. sputorum* is available in the public databases, and the species carries a complete capsule biosynthesis locus with high similarity to the polysaccharide capsule gene cluster of *H. influenzae*. Among *Haemophilus* and *Aggregatibacter* species with host specificity for humans, polysaccharide capsule biosynthesis loci have been documented only for *H. influenzae* and *H. sputorum*.

Haemophilus ducreyi

H. ducreyi is not closely related to other *Haemophilus* species (100, 103). The species is dependent on X factor but not V factor (Table 3), as the *nadV* gene that confers independence of V factor is located on a plasmid (104). The full-genome-sequenced *H. ducreyi* strain 35000HP carries both the extrachromosomal plasmid and tandem copies of the plasmid integrated into the genome (105). Strains of the species are fastidious and demonstrate little enzymatic activity in standard tests, including acid production from carbohydrates (Table 3). When cultured directly from infections, small yellow-gray colonies are observed that typically remain cohesive when pushed across the agar (106). *H. ducreyi* is distinct from other *Haemophilus* and *Aggregatibacter* species by 16S rRNA and housekeeping gene sequences (Fig. 1). Delineation of *H. ducreyi* by MALDI-TOF mass spectrometry appears to be robust (Table 5).

THE GENUS *AGGREGATIBACTER*

The genus *Aggregatibacter* was created to accommodate species that had previously been classified in the genera *Actinobacillus* (*Actinobacillus actinomycetemcomitans*) and *Haemophilus* (*Haemophilus aphrophilus*, *Haemophilus paraphrophilus*, and *Haemophilus segnis*); furthermore, *H. paraphrophilus* was shown to be a growth variant of *H. aphrophilus* (8). These species were only distantly related to the type species of their former genera but were sufficiently related to each other by 16S rRNA gene sequence, MLSA, and DNA hybridization to warrant creation of a new genus. The species of the genus *Aggregatibacter* are nonhemolytic and capnophilic; however, isolates of *A. actinomycetemcomitans* with overexpression of leukotoxin may exhibit a zone of hemolysis. There is no dependence on X factor, and the requirement for V factor is variable. Granular growth in broth is common and was noted in the original descriptions of "*Bacterium actinomycetem comitans*" (107) and *Haemophilus aphrophilus* (108). The generic name of the group was proposed to designate a rod-shaped bacterium that aggregates (8). Phenotypic characteristics differentiating the three described species of *Aggregatibacter* from other *Pasteurellaceae* species are shown in Table 3.

Aggregatibacter actinomycetemcomitans

Bacterium actinomycetem comitans was described in 1912, by Klinger (107), as a coccobacillary bacterium isolated together with *Actinomyces* from actinomycotic lesions of humans. This bacterium has undergone many nomenclatural changes: it was reclassified as *Actinobacillus actinomycetemcomitans* in 1929 (109), as *Haemophilus actinomycetemcomitans* in 1985 (110), and as *Aggregatibacter actinomycetemcomitans* in 2006 (8). It grows poorly in ambient air but well in 5% CO_2. Colonies on chocolate agar are small, with a diameter of ≤0.5 mm after 24 h, but their diameter may exceed 1 to 2 mm after 48 h. On primary isolation, the colonies are rough and adherent and demonstrate an opaque pattern described as star-like or like "crossed cigars" (111). The rough phenotype and the formation of biofilm are related to expression of long filamentous fibrils and production of poly-*N*-acetylglucosamine (112–114). Cells from rough colonies grow in broth as granular, autoaggregated cells that adhere to the glass and leave a clear broth. Successive rounds of *in vitro* subculturing on solid media can result in transformation of rough colonies into smooth, nonadherent colony types that exhibit planktonic growth in broth and a reduced ability to colonize the mouth of experimental animals (115). The rough-to-smooth conversion of *A. actinomycetemcomitans in vitro* is commonly, but not exclusively, caused by mutations of the *flp* promoter (116).

Six serotypes of *A. actinomycetemcomitans* (serotypes a to f) have been described. In contrast to the capsular polysaccharide-based antigenicity of serotypable *H. influenzae*, the serologic specificity of *A. actinomycetemcomitans* is defined by six structurally

and antigenically distinct O-polysaccharide components of the respective lipopolysaccharide molecules (117–120).

Salient biochemical characteristics of *A. actinomycetemcomitans* have been presented previously (121); key characters for discrimination between *A. actinomycetemcomitans* and V-factor-independent strains of *A. aphrophilus* are catalase and *o*-nitrophenyl-β-D-galactopyranoside (ONPG), plus fermentation of lactose and sucrose (Table 3). *A. actinomycetemcomitans* is distinct from other *Haemophilus* and *Aggregatibacter* species by 16S rRNA and housekeeping gene sequences (Fig. 1) (6, 8, 100). Delineation of *A. actinomycetemcomitans* by MALDI-TOF mass spectrometry appears to be robust (Table 5).

Aggregatibacter aphrophilus

Haemophilus aphrophilus was described as a cause of infective endocarditis in 1940 (108); the specific epithet (Gr. *aphros*, froth) denotes a requirement for elevated levels of CO_2. The close phenotypic relationship with *A. actinomycetemcomitans* was already noted by 1962 (122). *Haemophilus paraphrophilus* was later described as a species with a high level of resemblance to *Haemophilus aphrophilus*, but differing in growth factor requirements (dependence on V factor) (123). However, the V-factor-dependent phenotype is caused by a partial deletion of the gene encoding nicotinamide phosphoribosyltransferase, and the NAD-dependent phenotype is naturally reversible in strains competent for transformation (8). *Haemophilus paraphrophilus* is therefore a later heterotypic synonym of *Haemophilus aphrophilus*. With the unification of the two species, *A. aphrophilus* thus encompasses both V-factor-dependent and -independent isolates, but otherwise the species is phenotypically homogenous (Table 3). Key phenotypic characters for discrimination between V-factor-independent strains of *A. aphrophilus* and *A. actinomycetemcomitans* are catalase and ONPG, plus fermentation of lactose and sucrose; the key test for discrimination between V-factor-dependent strains of *A. aphrophilus* and *A. segnis* is fermentation of lactose (Table 3). A single study claimed a successful separation of *Haemophilus aphrophilus* from *Haemophilus paraphrophilus* based on 16S rRNA gene sequences (124). This finding was probably caused by the inclusion of strains of *H. parainfluenzae* erroneously identified as *Haemophilus paraphrophilus*; indeed, one of the original three strains of *Haemophilus paraphrophilus* (strain ATCC 29242) is a misidentified strain of *H. parainfluenzae* (8). Rather, 16S rRNA gene sequences from isolates of *A. aphrophilus* are homogenous and distinct from those of other species (8, 100, 125).

A. aphrophilus can also be delineated by housekeeping gene sequencing; however, analyses of gene fragments have indicated an unexpectedly high level of interspecies horizontal gene transfer (8), necessitating a multilocus approach to overcome the distorting effect of recombination at single gene loci. Delineation of *A. aphrophilus* by MALDI-TOF mass spectrometry appears to be robust (Table 5), but current databases need to be extended (126).

Aggregatibacter segnis

A. segnis was originally described as a species of *Haemophilus* characterized by slow growth and weak carbohydrate fermentation (L. *segnis*, sluggish) (28). The species was transferred to the genus *Aggregatibacter* in 2006 (8). Growth in broth and fermentation media is slow. The species is invariably dependent on V factor, while CO_2 enhances growth for some strains. Only quantitative differences in the amount of acid produced from carbohydrates can phenotypically differentiate *A. segnis* from strains of *H. parainfluenzae* biotype V (negative for tryptophanase, urease, and ornithine decarboxylase). *A. segnis* can be identified accurately by 16S rRNA gene sequencing (6, 8, 100). *A. segnis* can also be delineated by housekeeping gene sequencing; however, analysis of gene fragments from *A. segnis* has indicated a high level of interspecies horizontal gene transfer (8), necessitating a multilocus approach. Delineation of *A. segnis* by MALDI-TOF mass spectrometry appears to be robust (Table 5).

LABORATORY METHODS

Assignment to species by phenotype has been the standard method of identification for more than a century, but phenotypic testing has for some years been supplemented by DNA-based methods in reference laboratories. The new potent technique of MALDI-TOF mass spectrometry holds promise for altering routine identification in the future.

Assessment of Growth Factor Dependence

The members of the *Pasteurellaceae* are obligate parasites adapted to living on mucosal surfaces. They are propagated *in vitro* on rich media, such as chocolate agar, in which sheep or horse blood is added to a basic medium at a temperature of approximately 70°C. Members of the genus *Aggregatibacter* are capnophilic, and primary isolation may require the presence of elevated levels of CO_2. The detection of capnophilia is not a dependable criterion for differentiation between species; rather, the general use of 5% CO_2 can be utilized to sustain optimal growth of clinical isolates of *Haemophilus* and *Aggregatibacter* (8, 15, 127).

Dependence on X factor can be demonstrated by biochemical tests or by growth around paper disks impregnated with hemin chloride on agar plates devoid of X factor. Demonstration of X factor dependence using growth tests may carry a high rate of misinterpretations due to trace amounts of heme present in the medium or to heme inadvertently carried over with the inoculum (26, 48). Detecting the formation of porphyrins from δ-aminolevulinic acid performs better than growth-based testing methods and has become the standard method to document independence of X factor (27, 128, 129).

Dependence on V factor is conveniently demonstrated by the satellite growth of the investigated strain around a streak or colony of a "feeder" strain supplying the critical factor in excess. V-factor-dependent species of *Haemophilus* and *Aggregatibacter* grow to sizeable colonies immediately adjacent to the feeder strain, with decreasing colony sizes dependent on the distance (0.5 to 2 cm) from the feeder strain. An alternative method is to use a paper disk impregnated with NAD; nicotinamide mononucleotide and nicotinamide riboside can also serve as V factor (31). The demonstration of satellitism is robust, but the test is strictly dependent on the absence of V factor from the medium. Cultivation of *Haemophilus* and *Aggregatibacter* is usually done on complex media, and if NAD or certain NAD precursors are present in the formulations, a strain will not reveal its V factor dependence. Furthermore, if the test of satellitism is done on blood agar plates, hemolytic *Haemophilus* strains can obtain NAD from the lysed erythrocytes and grow to sizeable colonies over the entire agar (Fig. 4B). The V factor dependence of such strains can be demonstrated in the absence of blood (Fig. 4A) or by use of autoclaved media, in which NAD and precursors have been degraded completely.

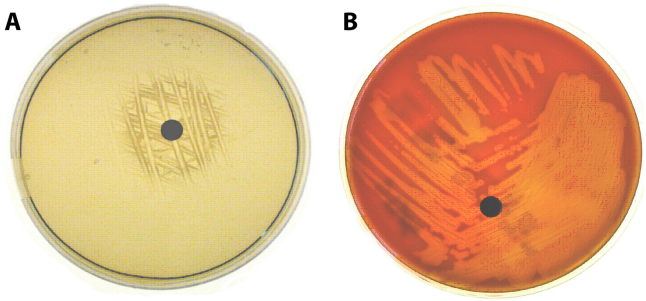

FIG 4 The type strain of *H. pittmaniae* was cultured for 24 h on different agars in the presence of a NAD-containing disk and photographed by transillumination. (A) Todd-Hewitt agar. Growth is apparent only around the NAD disk. (B) Five percent horse blood agar. Growth of hemolytic colonies is visible over the entire agar. (Reprinted from reference 15 with permission of the publisher.)

Other Phenotypic Characterization

Key phenotypic characteristics for differentiation of *Haemophilus* and *Aggregatibacter* species and for discrimination from species of *Actinobacillus* and *Pasteurella* isolated from human infections are given in Table 3 (15, 48, 99, 121, 127). Except for production of IgA protease, and possibly that of acid from carbohydrates, these phenotypic tests are readily available and are still the cornerstones of routine identification in many clinical microbiology laboratories. A great aid, almost a prerequisite, for identification in the routine laboratory is an exact knowledge of the clinical settings in which the various species of these genera occur (see below).

Phenotypic characteristics can be determined by conventional testing in single tubes (15, 28, 48, 130) or by a number of semi- or fully automated systems (129, 131–137). The use of automated systems is feasible and cost-effective, and they usually perform well when challenged with strains of species that are included in their databases (135–137). However, attempts to identify species that are not included in the identification database may lead to misidentifications (14, 138), which is highly unsatisfactory. Conventional testing in single tubes is flexible but time-consuming and is dependent on skilled staff, and the cost of extended phenotypic testing in single tubes is prohibitive for routine use. However, the rational use of selected phenotypic tests to confirm or reject a presumptive identification constitutes a powerful tool in the hands of an experienced microbiologist.

Production of tryptophanase (indole test), urease, and ornithine decarboxylase (ODC) is variable in *H. influenzae* and *H. parainfluenzae* (Table 4) and has been used for biotyping (28). The division of isolates into eight biotypes has limited discriminatory power for typing purposes, but the division may serve as an initial framework for preliminary specific assignment of X-factor-independent isolates: (i) strains that are negative in the three tests can be assigned to *H. parainfluenzae* biotype V, but this is also characteristic for the hemolytic species *H. pittmaniae* and the nonhemolytic *Aggregatibacter* species; and (ii) strains that are positive only for urease can be assigned to *H. parainfluenzae* biotype III, but this is also characteristic for the hemolytic species *H. parahaemolyticus* and *H. sputorum*, as well as the X- and V-factor-independent and nonhemolytic species *Actinobacillus ureae* and *Actinobacillus hominis*.

The viability of *H. ducreyi* is lost after 24 h in transport media at room temperature but may be preserved for several days at 4°C (139). It is recommended to inoculate clinical material directly onto the culture media and to incubate samples at 33°C in a humidified atmosphere with 5% CO_2 for a minimum of 48 to 72 h (140, 141). To suppress the resident flora of skin, the selective media for isolation of *H. ducreyi* usually incorporate vancomycin at a concentration of 3 mg/liter. Different strains of *H. ducreyi* may grow preferentially on different culture media, and the use of more than one type of media is recommended. Medium supplements include hemoglobin, fetal calf serum, chocolatized horse blood, and chemically defined growth-promoting substances, such as IsoVitaleX. The diagnostic tests for chancroid have been reviewed elsewhere (142).

DNA Sequencing

16S rRNA gene sequencing. Amplification of the 16S rRNA gene by PCR, followed by sequencing and comparison with deposited sequences in the public databases, is a powerful technique for identifying *Haemophilus* and *Aggregatibacter* species. Primers described for other *Gammaproteobacteria* (143–146) usually work well with the *Pasteurellaceae* (15, 147, 148). The diversity of 16S rRNA genes is sufficient to assign an unknown strain to a genus, and usually also to a species, on the basis of a 500-nucleotide (nt) sequence arising from a single sequencing reaction, but the discriminatory power may be insufficient for separation of *H. influenzae* from *H. haemolyticus* and related organisms (36, 37). Furthermore, the differentiation of *Haemophilus aegyptius* from *H. influenzae* and of *Haemophilus parahaemolyticus* from *Haemophilus paraphrohaemolyticus* is hampered by the taxonomic uncertainties regarding *H. aegyptius* and *H. paraphrohaemolyticus*. 16S rRNA gene sequencing can be impeded by polymorphic nucleotide positions resulting from intragenomic heterogeneity between the multiple rRNA genes. Six copies of the 16S rRNA gene are usually present in the genomes of *Haemophilus* and *Aggregatibacter* (41, 149–152), but little 16S rRNA gene heterogeneity is observed in these genomes. Recently, an unexpected and conspicuously large number of polymorphic positions was observed in a collection of *H. haemolyticus* strains and related organisms (see below) (153), and such a degree of heterogeneity may seriously interfere with 16S rRNA gene-based identification.

Housekeeping gene sequencing. Different protein-coding genes have been studied and compared for species of *Haemophilus* and *Aggregatibacter* (8, 98, 101, 154–157). The increased sequence variation of translated genes compared to rRNA genes confers more information for analysis, but this must be weighed against the increased probability of prior recombination events affecting the genes. In a study of housekeeping genes including nine strains of *Aggregatibacter*, 3 of 36 (8%) gene fragments in *Aggregatibacter* had been subject to recombination across the species barrier (98). This observation was expanded upon by examination of a larger collection of *Aggregatibacter* strains, where recombination across the species barrier was observed for 12 of 40 *infB* fragments, 5 of 40 *recA* fragments, and 0 of 40 *pgi* fragments. Recombination across the genus barrier was found to presumably occur for *infB*, where sequences with high similarity to the corresponding gene in *H. parainfluenzae* were observed in some isolates of *A. segnis* (8). Identification to the species level based on partial sequencing of a single housekeeping gene may thus be erroneous. To overcome the distorting effect of recombination at single gene loci, it is necessary to investigate a number of separate genes and to perform the analysis on concatenated sequences (MLSA).

Identification by PCR

PCR-based detection of *Haemophilus* and *Aggregatibacter* in clinical material has focused mainly on rapid diagnosis of *H. influenzae* meningitis (158–161), improved detection of *H. ducreyi* (142), and identification of the virulent clone JP2 of *A. actinomycetemcomitans* (162, 163). PCR has also been utilized for detection of *H. influenzae* DNA in culture-negative middle ear fluids where prior antibiotic therapy has made culturing inconclusive (164) and for improved microbiological surveillance of bacterial meningitis in parts of the world where laboratory facilities for immediate culturing of cerebrospinal fluid samples are not available (165). With respect to *H. influenzae* meningitis, the development of PCR technology coincided with the implementation of the Hib vaccine, which, on one hand, reduced the need for an assay targeting *H. influenzae* due to reduced incidence of the disease. But on the other hand, the need for surveillance of Hib vaccine efficacy and of serotype replacement still made correct identification mandatory. The most commonly used assay has probably been the dual-target PCR approach of van Ketel et al., which specifically targets capsulated strains (160). One primer set was specific for the capsule export protein gene *bexA* and amplified target DNAs from *H. influenzae* strains of all serotypes, while the other primer set recognized the gene encoding the outer membrane protein P6. The latter set also amplified DNAs from *H. haemolyticus* and two of nine *H. parainfluenzae* strains (160). Failure to detect the capsule export protein gene with the *bexA* primers in *H. influenzae* serotypes e and f was subsequently reported (69, 166, 167). Serotype e and f strains belong to phylogenetic group II of *H. influenzae*, which encompasses relatively distant lineages of the species. Other targets for PCR detection of capsulated strains include *bexB* (168), present in all serotypes, and the serotype b-specific polysaccharide-synthesizing gene *bcs3* (169). The newly described species *H. sputorum* contains a complete capsule biosynthesis locus with high similarity to the capsule gene cluster in *H. influenzae*, and standard *bexA* and *bexB* PCRs give positive results with strains of *H. sputorum* (unpublished observation). Thus, positive results with current *bexA* and *bexB* PCRs are not specific for *H. influenzae* and must be confirmed by other means. With the implementation of Hib vaccination, non-serotype b and unencapsulated strains have gained importance as causes of *H. influenzae* infections. For detection of all *H. influenzae* strains, regardless of the encapsulation status, other targets have been tested, including the 16S rRNA gene (164, 170, 171), *ompP2* (63), *ompP6* (172), and *hpd* (161, 173). PCR amplification of specific marker genes to discriminate *H. influenzae* from neighboring taxa is described below.

The difficult culture of *H. ducreyi* from clinical specimens makes it an ideal candidate for detection by molecular techniques. Primer sets targeting the 16S rRNA gene (174–176), the ribosomal intergenic spacer region (177), *recD* (178, 179), and the heat shock protein gene *groEL* (179, 180) have been published.

MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectrometry has emerged as a rapid and accurate means of identifying microorganisms by separating peptides and proteins from cells according to mass (181–184). A spectrum representing the released molecular fragments is generated within minutes, and identification is accomplished by comparison with reference spectra in a database. Two separate strategies have been developed for identification: either inclusion of a large number of spectra for each taxon in the database or generation of artificial spectra incorporating only taxonomically important peaks, i.e., peaks representing peptide components present in the majority of strains of a species and absent from related taxa (185, 186). Identification by MALDI-TOF mass spectrometry of HACEK (*Haemophilus* and *Aggregatibacter* spp., *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*; see below for more details) clinical isolates, including *H. influenzae*, *H. parainfluenzae*, *A. actinomycetemcomitans*, and *A. aphrophilus*, was recently reported (126). Despite the use of modest thresholds of identification (log scores of >1.7 for reliable genus identification and >1.9 for reliable species identification), only 93% of isolates were correctly identified to the genus level, and 66% to the species level. Of the *Haemophilus* and *Aggregatibacter* species tested, *A. aphrophilus* isolates produced mainly genus-level identifications, and one isolate was misidentified as *H. influenzae* (126). Clinical *A. aphrophilus* isolates showed notable spectral differences compared to the single reference database entry, and the average score for *A. aphrophilus* increased significantly by use of a customized database incorporating a local clinical isolate (126).

Other mass spectrometry investigations of *Haemophilus* and *Aggregatibacter* have found large differences between spectra obtained from the majority of the species (99, 187). Table 5 lists average similarity scores for mass spectra of two or three selected test strains compared with type strains of species. Representatives of unnamed taxa related to *H. haemolyticus* ("cryptic genospecies biotype IV," "nonhemolytic *H. haemolyticus*," and *Haemophilus intermedius*) are combined into a group designated "cryptic genospecies." The mass spectrometry analysis separated species robustly, with two exceptions: there was insufficient resolution within the *H. influenzae* group (*H. influenzae*, *H. aegyptius*, *H. haemolyticus*, and "cryptic genospecies") and between *H. paraphrohaemolyticus* and *H. parahaemolyticus*. The spectral differences presented in Table 5 indicate that the databases and identification algorithms of MALDI-TOF mass spectrometry can be expanded and refined, which will enable reliable identification of the large majority of human *Haemophilus* and *Aggregatibacter* isolates. Some issues and questions remain, such as the use of a single artificial spectrum versus multiple spectra for each taxon (185, 186) and whether databases should be modified locally by incorporation of spectra from reference strains that have been cultured, extracted, and analyzed in the same manner as clinical isolates (126). Because of the close relationship and taxonomic uncertainties regarding some of the species in the genus *Haemophilus*, MALDI-TOF mass spectrometry cannot be expected to definitively identify all isolates from this genus. But the ease, speed, and precision of the procedure will undoubtedly result in improved routine identification of the more rarely encountered microorganisms from clinical specimens.

Other Methods

Fluorescence *in situ* hybridization (FISH) with probes specific for *H. influenzae* DNA has been used to identify the bacterium when Gram-negative bacilli are seen on microscopy of cerebrospinal fluid (188) and to detect *H. influenzae* DNA in adenoid biofilms of otitis-prone children (189) and in the mucosae of patients with chronic rhinosinusitis (190). Use of microarray hybridization for identification has been introduced for profiling the oral microbiotas of individuals (191, 192) but has not yet evolved as a useful method for routine identification.

Differentiation of *Haemophilus influenzae* from *Haemophilus haemolyticus*

The recognition of the inadequacy of current methods for the differentiation of *H. influenzae* from nonhemolytic variants of *H. haemolyticus* has prompted a large number of investigations addressing either the magnitude of misidentifications or the ability of an assay or an algorithm to confirm or reject a putative identification of *H. influenzae*. The major challenge in the evaluation of these studies is the lack of a universal delineation of *H. influenzae*. Various collections of strains are investigated and different tests are used to define *H. influenzae*; thus, isolates that are included in the species in one investigation may be excluded from the species in another. Categorizations such as "equivocal" (88) and "fuzzy species" (173) are legitimate descriptions of diagnostic uncertainties, but the variable definition and use of such terms are further complications.

Phenotypic traits. *H. influenzae* is nonhemolytic and dependent on both the X and V factors. Plasmids from *H. ducreyi* are capable of conferring NAD independence on *H. influenzae* in the laboratory (104), but exceptions to the invariable dependence on both the X and V factors and the absence of hemolysis have not been reported for naturally occurring *H. influenzae* to date (36, 37, 86). *H. influenzae* usually ferments both ribose and xylose and does not ferment sucrose or mannose (36, 48). Expression of a functional IgA1 protease is considered a specific trait of only three *Haemophilus* species, i.e., *H. influenzae*, *H. aegyptius*, and *H. parahaemolyticus* (48, 193), but it was recently shown that several isolates of *H. haemolyticus* and related organisms, including representatives of the X-factor-independent *Haemophilus intermedius*, specifically cleaved IgA1 and were positive for the encoding gene (*iga*) by hybridization (36). The presence of a functional IgA1 protease in strains which clearly do not belong to *H. influenzae* challenges the specificity of this phenotypic trait, while it does not exclude the possibility that conserved regions of the *iga* gene may be used in PCR or hybridization assays to distinguish strains of *H. influenzae* from related taxa (see below).

Production of gas from fermentation of glucose, emission of H_2S, or conformational changes in the outer membrane protein (OMP) P6 are other phenotypic traits that have been exploited for differentiation within the *H. influenzae* group (Table 6). A monoclonal antibody (MAb 7F3) has been shown to react with OMP P6 of *H. influenzae* but not with those of strains of *H. haemolyticus* or cryptic genospecies biotype IV (86, 194). However, the specificities of MAb 7F3 and the other differentiating phenotypic traits are limited. In a study of a large number of strains classified by a five-gene MLSA, production of gas was observed for 6% of *H. influenzae* and 88% of *H. haemolyticus* strains, production of H_2S was observed for 13% of *H. influenzae* and 69% of *H. haemolyticus* strains, and MAb 7F3 reacted with 97% of *H. influenzae* and 12% of *H. haemolyticus* strains (37). It should be emphasized that this study addressed primarily carriage isolates, i.e., a population where a large proportion of isolates with aberrant test results must be expected.

16S rRNA gene sequencing. Analysis of complete or near-full-length 16S rRNA gene sequences (1.4 kb) segregates X- and V-factor-dependent *Haemophilus* strains into distinct groups that cluster with the type strain of either *H. influenzae* or *H. haemolyticus* (36, 86, 195, 196) (Fig. 5). However, modest bootstrap support of the 16S rRNA gene clusters signifies a risk of misclassifications (36, 196), and this risk is increased when analysis is based on shorter 16S rRNA gene fragments. Indeed, the segregation of strains into two clusters by an 886-nt 16S rRNA gene fragment was in conflict with the clustering based on a five-gene MLSA (37). Caution must therefore be exercised if 16S rRNA gene sequencing is used as the gold standard for the delineation of *H. influenzae*. In comparison with MLSA, the inferior resolution of the 16S rRNA gene analysis is caused by less variability, a shorter fragment length, and the presence of polymorphic positions in the multiple copies of the 16S rRNA gene. An unexpectedly high level of 16S rRNA gene polymorphism was recently described for a collection of strains of *H. haemolyticus* and related organisms (153). The average frequency of 16S rRNA gene polymorphic nucleotide positions was approximately 10 times the level observed in *H. influenzae*. Up to 36 polymorphic positions in the 16S rRNA gene of a single strain were observed, corresponding to 2.6% of the positions in the sequenced fragment (1,362 nt). For comparison, the type strain of *H. haemolyticus* shows 96.9% identity with the type strain of *H. influenzae*. The outer stem of 16S rRNA gene helix 18 is an illustrative example (Fig. 6). Three distinct types of helix 18 were present in the collection, represented by *H. influenzae* strain Rd, cryptic genospecies biotype IV strain 16N, and *H. haemolyticus* strain NCTC10659^T (Fig. 6). However, seven other strains harbored two separate helix 18 types; depending on the combination, the mixture of helix types resulted in 8, 9, or 11 polymorphic positions within the region of 23 nt that comprises the outer stem of helix 18 (153). These findings emphasize that even rRNA genes may be subject to interspecies recombination among members of the *H. influenzae* group.

Multilocus sequence analysis. The most reliable delineation of *H. influenzae* at present is based on concatenated sequences of housekeeping gene fragments, using either the *H. influenzae* MLST scheme (43) or an alternative, five-gene scheme that includes a fragment of the 16S rRNA gene (37). The alternative scheme is based on a general MLSA developed for the family *Pasteurellaceae* (98), but the primers were improved to specifically

TABLE 6 Tests differentiating *H. influenzae* from *H. haemolyticus* and related organisms

Characteristic	Method	References
Production of gas from glucose	Phenotype	36, 37
H_2S emission	Phenotype	36, 37
IgA1 cleavage	Phenotype	36
OMP P6 conformation	Immunoassay	37, 86, 194
Detection of:		
fucK	Hybridization, PCR	36, 86, 88, 173, 196, 199, 206
hap	PCR	36, 199
hpd	PCR	88, 161, 173, 196
iga	Hybridization, PCR	36, 37, 85, 173
lgtC	Hybridization	37, 173
ompP2	PCR	161, 173
ompP6	PCR, sequencing	160, 172, 173, 200
pilA	PCR	299
rrs[a]	PCR	84, 86, 173
sodC	Hybridization, PCR	36, 88, 95, 196, 199, 202, 203, 206

[a] See the text for information on differentiation by 16S rRNA gene sequence comparison.

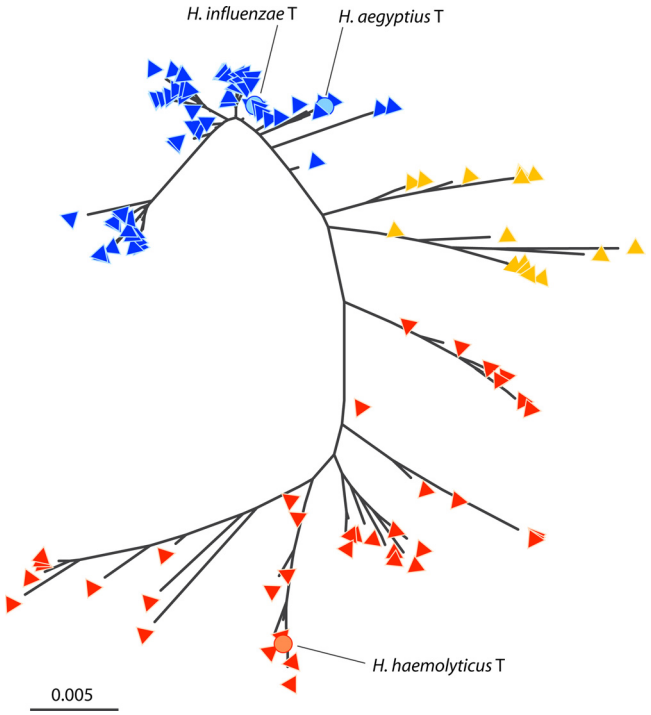

FIG 5 Neighbor-joining tree based on near-full-length 16S rRNA gene sequences (1,361 or 1,362 nucleotides), comparing the type strains of *H. influenzae*, *H. aegyptius*, and *H. haemolyticus* (filled circles) with 80 strains of *H. influenzae* (36, 199, 296) and 39 reference strains of *H. haemolyticus* and related organisms (36, 195, 199, 294) (filled triangles). Strain PN134 was omitted from the comparison because of doubtful identification to the species level (199). Phylogenetic group I is indicated in blue, phylogenetic group II in yellow, and *H. haemolyticus* and related organisms in red. *H. haemolyticus* and related organisms are located on two branches, with one adjacent to *H. influenzae* phylogenetic group II and composed mainly of non-hemolytic *H. intermedius* subsp. *gazogenes* (bootstrap support, 36%) and a larger cluster encompassing the type strain, porphyrin-synthesizing strains, and cryptic genospecies biotype IV strains (bootstrap support, 57%). In contrast, all representatives of *H. influenzae* and the type strain of *H. aegyptius* are located in a single cluster supported by a bootstrap value of 63%. Analysis was conducted using MEGA5 (295), and ambiguous positions were removed for each sequence pair.

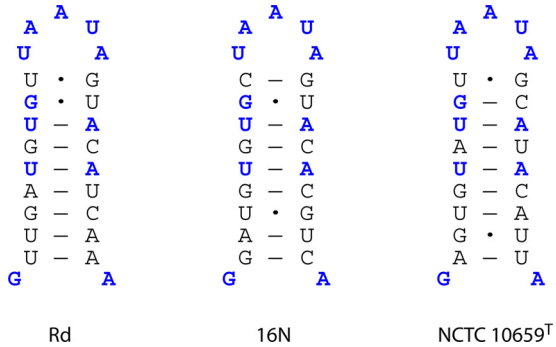

FIG 6 The three 16S rRNA gene helix 18 types present in a collection of isolates from the *H. influenzae* group, represented by *H. influenzae* strain Rd, cryptic genospecies biotype IV strain 16N, and *H. haemolyticus* strain NCTC 10659^T. The outer stem of helix 18 (nt 453 to 477) is shown. Conserved nucleotides are shown in blue. (Adapted from reference 153 with permission of the publisher.)

target the *H. influenzae* group. If the *H. influenzae* MLST scheme is employed, the fuculokinase gene *fucK* is omitted because this gene is usually not present in *H. haemolyticus* (36, 86, 197). Figure 3 depicts a comparison of 900 *H. influenzae* sequence types from the MLST website with the type strains of *H. influenzae*, *H. aegyptius*, and *H. haemolyticus* plus 35 isolates of *H. haemolyticus* and related organisms that have been excluded from *H. influenzae*. The type strain of *H. haemolyticus* and the 35 non-*influenzae Haemophilus* reference strains are located in a common cluster (red in Fig. 3) that is clearly separate from phylogenetic groups I (blue) and II (yellow) of *H. influenzae*. Also included in the *H. haemolyticus* cluster are seven sequence types from the MLST website: ST35, deposited as a cryptic genospecies biotype IV strain, plus ST743, ST759, ST815, ST816, ST845, and ST911, which may represent misidentified strains of *H. haemolyticus* or related organisms. The modest bootstrap support of the red cluster in Fig. 3 (67%) is caused by ST743, ST759, and ST816; if these three STs are omitted from the comparison, the bootstrap support of the red cluster increases to 99% (not shown).

Detection of biomarker genes by PCR. A number of genes have been explored for use as targets in the differentiation of *H. influenzae* from *H. haemolyticus* (Table 6). Some of the biomarker genes, such as *fucK* and *sodC*, are either present or absent in the genomes of the members of the *H. influenzae* group (although *sodC* may be present as a pseudogene). In this case, there is more flexibility for design of amplification and/or hybridization conditions. For other biomarkers, such as *hpd* and *ompP6*, homologs of the genes are probably present in all strains of the *H. influenzae* group, and differentiation is linked to preserved nucleotide motifs within the target genes. In the latter case, strict adherence to published protocols is essential for interpretation and comparison of results.

Fuculokinase is one of four enzymes involved in the fucose pathway (198), and the encoding gene, *fucK*, is one of seven genes included in the MLST scheme for typing of *H. influenzae* (43). Murphy and coworkers were unable to amplify the gene from variant strains (86), suggesting *fucK* as a favorable marker of *H. influenzae* (36). *fucK* was indeed superior to *sodC* and *hap* in an assessment of marker genes for identification of misidentified strains among 480 unselected clinical isolates of *H. influenzae* (199). The presence of *fucK* has not been assessed in a large collection of *H. haemolyticus* isolates. The gene cannot unambiguously identify *H. influenzae*, as occasional strains of *H. influenzae* are negative for *fucK* due to a complete or partial deletion of the fucose operon (40, 196, 197); similarly, *fucK* sequences have been amplified successfully by PCR from occasional strains excluded from this species (173, 200). Because *fucK* is part of the MLST scheme, primers for amplification of the gene are widely available. For practical purposes, the failure to amplify the *fucK* fragment from a presumptive isolate of *H. influenzae* suggests an incorrectly identified strain that should be characterized further.

Two promising biomarker genes are *hpd*, encoding the surface-exposed lipoprotein protein D, and *iga*, encoding IgA1 protease. *hpd* is conserved among *H. influenzae* serotype b and unencapsulated strains (201). PCR assays targeting *hpd* for detection of *H. influenzae* meningitis did not amplify *hpd* sequences from 16 isolates of *H. haemolyticus* (161), suggesting that this assay could provide an additional tool for differentiating the two species; this was confirmed in several recent studies (173, 196). Detection of *iga* has also performed well in several studies (37, 85, 173). McCrea and coworkers found complete segregation of *iga* gene probe hy-

bridization in an MLSA-based dendrogram, where 109 *iga*-negative, putative *H. haemolyticus* strains clustered separately from 88 *iga*-positive strains, including reference strains of *H. influenzae* (37). As stated above, some variant strains excluded from *H. influenzae* express a functional IgA1 protease, and probes generated from different regions of the *iga* gene show variable hybridization with a panel of test strains (36). It is therefore crucial to standardize template DNAs used for generation of hybridization probes and to identify optimal primers and conditions when PCR-based assays are employed for detection of *iga*.

In addition to antibody recognition of outer membrane protein P6, the encoding gene, *ompP6*, has been investigated by PCR (172, 173) and sequencing (200). Residues 33, 42, 59, and 61 of OMP P6 are alanine, alanine, aspartate, and threonine, respectively, in *H. influenzae*, while the corresponding residues in *H. haemolyticus* are glycine, serine, asparagine, and glutamate; in particular, the conformation of the MAb 7F3 epitope depends on amino acids 59 and 61 (194). Chang and coworkers sequenced the *ompP6* genes of 163 isolates obtained from the pharynxes of healthy children and from cases of pediatric otitis media (200). Based on translated amino acid sequences at the four key residues, all otitis and nasopharyngeal isolates were classified as *H. influenzae*, while 12 of 63 oropharyngeal isolates were *H. haemolyticus*. However, six additional isolates could not be categorized based on *ompP6* sequencing; when these isolates were subjected to MLST, the *adk* gene could not be amplified for two isolates, while the four remaining isolates clustered with *H. haemolyticus* (200). Additionally, 8 of 163 isolates had variations in the OMP P6 translated amino acid sequence at sites outside the four key residues; 2 of these isolates were subjected to MLST and finally identified as *H. influenzae* and *H. haemolyticus*. It was concluded that molecular characterization of *ompP6* was unable to differentiate all strains of *H. influenzae* from *H. haemolyticus* (200).

The copper-zinc-cofactored superoxide dismutase (CuZnSOD) encoded by *sodC* is present in cryptic genospecies biotype IV and in *H. haemolyticus* and has been used for differentiation of these taxa from *H. influenzae* (95, 202). Recent hybridization studies confirmed the presence of *sodC* in all investigated strains of *H. haemolyticus* and related organisms (36, 203). However, a *sodC* homolog encoding an inactive CuZnSOD enzyme is present in capsulated phylogenetic group II strains of *H. influenzae* (204), in which the gene is located adjacent to the capsule export protein gene *bexA* (57, 205). *sodC* may also be present in unencapsulated strains of *H. influenzae* (203, 206); interestingly, an active CuZnSOD enzyme was detected in the latter strains, which may indicate acquisition through interspecies recombination with *H. haemolyticus* (203). Low prevalences of *sodC* have been reported for clinical isolates of genuine *H. influenzae*, ranging from 1.3% among unselected clinical isolates (199) to 3.2% among isolates from patients with cystic fibrosis (206). In contrast, a *sodC* prevalence of 9.2% was demonstrated in a collection of unencapsulated *H. influenzae*, mostly composed of commensal, nonclinical strains (203). Consequently, detection of *sodC* cannot unambiguously discriminate between *H. influenzae* and *H. haemolyticus*.

Two recent studies evaluated and compared PCR screening assays for differentiation of *H. influenzae* from *H. haemolyticus* and related organisms among nasopharyngeal carriage isolates. One study screened 245 presumptive *H. influenzae* isolates for *fucK* and *hpd* and performed near-full-length (1,462 nt) 16S rRNA gene sequencing on 119 of the isolates (196). Another study characterized 60 selected strains from healthy and otitis-prone children by partial sequencing of 16S rRNA (598 nt) and *recA* (543 nt) genes and by seven separate PCR assays, including assays of *fucK*, *hpd*, *iga*, and *lgtC* (173). For identification of *H. influenzae*, the *hpd* PCR performed with a high sensitivity (88% and 89% for the healthy and otitis-prone children, respectively). Both studies found the *fucK* PCR to perform with reduced sensitivity, with 37% and 24% of study-defined *H. influenzae* strains, respectively, being negative for this biomarker gene. The investigation of carriage isolates from Minnesota did not detect *fucK* in 44 non-*influenzae Haemophilus* strains (196), while the study from Australia amplified this gene from 6 of 25 non-*influenzae Haemophilus* strains (173). However, it should be emphasized that different delineations of *H. influenzae* were employed in these studies. Whereas 119 strains could be assigned to two separate clusters by a single 1,469-nt 16S rRNA gene sequence (196), the diversity revealed by partial *recA* and 16S rRNA gene fragments precluded complete dichotomous identification of species; rather, isolates were interpreted as *H. influenzae* if they had approximately 97% DNA similarity (or higher) with the reference strain 86-028NP (16S rRNA and *recA* concatenated sequence) and possessed most of the target genes (PCR results) (173).

MALDI-TOF mass spectrometry. Two recent studies used MALDI-TOF mass spectrometry, on a single platform (207) or two separate platforms (208), to differentiate *H. haemolyticus* from *H. influenzae*. Direct comparison of the measured *H. haemolyticus* and *H. influenzae* spectra revealed high overall spectral similarities between the species, with considerable intraspecies variability (208). Both studies found the manufacturers' databases to be insufficient for distinction of species, and both studies successfully identified test strains after inclusion of suitable spectra in a modified database. Comparing strains against a database that encompasses some of the test strains is not scientifically correct; however, the results are indeed promising and should be expanded. A complete concordance of tests for *fucK* and OMP P6 with identification to the species level (207) indicates that the most challenging strains, in terms of identification, have yet to be subjected to the procedure.

CLINICAL SIGNIFICANCE

New knowledge on the difficult delineation of *H. influenzae*, the description of new species, and the taxonomic rearrangements of *Haemophilus* and *Aggregatibacter* have consequences for the clinical significance attributed to the species of these genera. The following focuses on these aspects and gives an update on the association of particular species with various clinical syndromes. A number of pertinent reviews are listed for more in-depth information.

Infectious Endocarditis and the HACEK Group

The so-called HACEK group of fastidious Gram-negative organisms is a recognized but unusual cause of infective endocarditis, responsible for 1.4 to 3% of cases (209, 210). The group was originally described to encompass *Haemophilus* species, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae* (211). In the original report, *Haemophilus* spp. accounted for 18 of 32 HACEK organisms involved, including two *H. influenzae* strains (211). A review of endocarditis due to rare and fastidious bacteria covering the years 1966 to 1999 reported that only 13 of 398 HACEK cases were caused by *H.*

influenzae (212), making this a rare cause of the infection. *H. influenzae* is a more common etiology of adult bacteremia than other *Haemophilus* and *Aggregatibacter* spp. combined (213); consequently, the isolation of *H. influenzae* from blood is rarely a sign of infective endocarditis, and the species is sometimes omitted from the HACEK group (126, 214). After the recent taxonomic rearrangements, the HACEK acronym can still be used for the group, with the acronym now denoting *Haemophilus* and *Aggregatibacter* spp., *C. hominis*, *E. corrodens*, and *K. kingae*. But the relative proportions have changed, with the genus *Aggregatibacter* now being the dominant etiology of HACEK endocarditis. The literature review covering 1966 to 1999 found 92 cases caused by *A. actinomycetemcomitans* and 99 by *A. aphrophilus* (reported as *Haemophilus aphrophilus* and *Haemophilus paraphrophilus*), in comparison to 66 cases caused by *H. parainfluenzae*, 13 by *H. influenzae*, and 128 by *C. hominis*, *E. corrodens*, and *K. kingae* combined (212). A multicenter study describing 77 cases of HACEK endocarditis during 2000 to 2006 found 31 cases caused by *Haemophilus* spp. and 26 by *Aggregatibacter* spp. (210); however, not all *Haemophilus* isolates were identified to the species level. *A. segnis*, which was not included in the original definition of HACEK organisms, is a rare cause of infective endocarditis (210, 215, 216).

The epidemiological and clinical features of infective endocarditis caused by *A. actinomycetemcomitans* (217) and *Haemophilus* (218) have been reviewed, and the characteristics and outcomes of HACEK endocarditis were recently published for a prospective, multinational cohort study (210). Extended incubation of blood cultures to increase the recovery of HACEK bacteria is considered unnecessary (213, 214).

Haemophilus influenzae in the Post-Hib-Vaccine Era

Vaccination against *H. influenzae* serotype b (Hib) was initiated in 1985, when polyribosylribitol phosphate polysaccharide vaccines were licensed for use. Conjugated vaccines with greatly improved immunogenicity in young children followed a few years later (219). In the pre-Hib-vaccine era, *H. influenzae* meningitis and epiglottitis were caused predominantly by serotype b strains and mainly affected children of ≤5 years of age (220–222). The implementation of Hib conjugate vaccines into routine vaccination schedules dramatically reduced the burden of invasive *H. influenzae* disease in many developed countries, while implementation of the vaccine in developing countries has progressed more slowly (219). In 2000, it was estimated that Hib caused 371,000 deaths worldwide in children of <5 years of age (223). Routine use of the Hib vaccine has changed the epidemiology of *H. influenzae*, and unencapsulated *H. influenzae* now accounts for most invasive infections, followed by serotypes f and b (224–229). An unexpectedly large proportion of invasive childhood infections caused by serotype a was recently reported from several Canadian provinces (205, 230) and among Alaska Native children (231). There is no evidence of substantial replacement disease with non-b serotypes in young children in the United States (232) or of increases of non-b infection in Australian indigenous children (233).

Although invasive *H. influenzae* disease was primarily a childhood disease in the prevaccine era, serious infections caused by unencapsulated strains were noted in adults (234, 235). The incidence of invasive *H. influenzae* disease may actually have increased in recent years (227, 229), but since Hib vaccine failure is rare (236), most invasive infections occur in the extreme ages of life and in patients with predisposing conditions (224, 226, 229, 232).

With the dramatic reduction of serious invasive disease caused by Hib, focus has shifted to less severe but far more prevalent infections caused by unencapsulated *H. influenzae* strains, such as otitis media, conjunctivitis, sinusitis, and exacerbation of chronic obstructive pulmonary disease. Readers are referred to reviews of *H. influenzae* and infections of the respiratory tract for more information (237–243).

The clinical features of Brazilian purpuric fever and the emergence and disappearance of the virulent BPF clone of *H. influenzae* have been reviewed previously (77).

Haemophilus haemolyticus and Related Organisms

Several lines of evidence indicate that the pathogenicity of *H. haemolyticus* is much reduced compared with that of *H. influenzae*. While 15 to 20% of presumptive *H. influenzae* nasopharyngeal isolates can be identified as *H. haemolyticus* and related organisms by molecular characterization (84, 85, 87), not a single one of these was detected among 130 middle ear fluid isolates obtained by means of tympanocentesis (86), supporting the view that *H. haemolyticus* is not a cause of otitis media. Reinvestigation of presumptive *H. influenzae* isolates cultured from lower respiratory tract samples from cystic fibrosis patients (206) or from unselected clinical samples submitted to the laboratory on suspicion of lower respiratory tract infection (199) detected <1% misidentified strains, which also points to a minor pathogenic role for *H. haemolyticus* and related organisms. In contrast, a characterization of presumptive *H. influenzae* isolates cultured from surveillance sputa from adults with chronic obstructive pulmonary disease revealed that almost 40% were *H. haemolyticus* (86). However, in the prospective part of that study, acquisitions of new strains of *H. haemolyticus* were not associated with pulmonary exacerbations, whereas 45% of acquisitions of new strains of *H. influenzae* were associated with exacerbations.

Recent data from the Centers for Disease Control and Prevention (CDC) emphasize that *H. haemolyticus* and related organisms should not be considered strict commensal organisms devoid of pathogenic potential. A retrospective characterization by near-full-length 16S rRNA gene sequencing of 374 invasive isolates of unencapsulated *H. influenzae* referred to the CDC revealed 7 isolates (1.9%) to be *H. haemolyticus* or related organisms (195). Isolates were recovered from blood in five cases and from synovial fluid and a pancreatic specimen in one case each; six of the seven patients had underlying medical conditions or recent surgical procedures. Five of the isolates were hemolytic, which categorically excludes identification as *H. influenzae*. The proportion of *H. haemolyticus* and related organisms among invasive strains (195) thus exceeded the reported proportion cultured from unselected clinical samples (199). A reliable method to distinguish *H. influenzae* from *H. haemolyticus* is a prerequisite for determining their specific clinical significance.

The cryptic genospecies biotype IV is recognized as a rare but definite neonatal, maternal, and urogenital pathogen (244). The majority of infections are associated with the female genitourinary tract and neonates (244–246); however, a series of cryptic genospecies biotype IV isolates cultured from urethral discharge or urine from adult males have also been reported (93).

Other *Haemophilus* Species

H. parainfluenzae is the most commonly found *Haemophilus* species in infective endocarditis (see above) and is the most commonly found non-*influenzae Haemophilus* species in bloodstream infections (213, 247). However, some reports of invasive infections with *H. parainfluenzae* may be misidentifications caused by erroneous assessment of X factor independence (222).

Margaret Pittman originally separated *H. parahaemolyticus* from *H. haemolyticus* and found the former species to be associated frequently with acute pharyngitis and occasionally with subacute endocarditis (82). *H. parahaemolyticus* has been observed as a dominant member of the cultivable microbiota in patients with pharyngitis (248), while being virtually absent from the oral cavity and pharynx in healthy children and adolescents. *H. parahaemolyticus* strains express IgA1 protease (48), which is capable of specifically cleaving and functionally inactivating human secretory IgA, which is the principal mediator of humoral immunity of the respiratory mucosa. Functional IgA1 proteases are also expressed by the important bacterial pathogens *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *H. influenzae* (249, 250). For this reason, *H. parahaemolyticus* has been considered a potential pathogen, but the evidence linking the species to acute pharyngitis is at present circumstantial.

H. pittmaniae was described in 2005, based on isolates cultured from saliva but also from various body fluids, without description of the patient cases (98). It was recently reported as responsible for respiratory tract infection in a patient with underlying lung disease (251), with the isolate identified by MALDI-TOF mass spectrometry. Both *H. pittmaniae* and *H. sputorum* are homogenous species (98, 99) characterized by distinct mass spectra (Table 5), and it can be presumed that their clinical significance will be clarified with improvement of databases and increased use of mass spectrometry. In contrast, *H. paraphrohaemolyticus* appears to be exceedingly rare in clinical specimens and cannot easily be identified (99).

Chancroid (soft chancre or ulcus molle) is a sexually transmitted disease caused by *H. ducreyi* and characterized by genital ulcerations accompanied by regional lymphadenitis and bubo formation. The disease is rare in developed countries, with only eight reported cases in 2011 in the United States (252). Its evolution (253), host-pathogen interaction (254), and clinical significance (255–257) have been reviewed. Genital ulceration is a major cofactor in the transmission of human immunodeficiency virus (256, 257).

Aggregatibacter actinomycetemcomitans and Adolescent Periodontitis

A. actinomycetemcomitans was originally coisolated with *Actinomyces* from actinomycotic lesions (107), and subsequent case reports of patients with infections in a variety of anatomical localizations have confirmed this association (258, 259). Among the *Actinomyces* species, coisolation of *A. actinomycetemcomitans* appears restricted to *Actinomyces israelii* (260); the molecular basis of this association is unknown. *A. actinomycetemcomitans* is also seen as a cause of infective endocarditis (see above) but has lately attracted attention because of its association with periodontitis (261–264). Longitudinal cohort studies have confirmed the significant connection between *A. actinomycetemcomitans* and the development of periodontitis (265–267). Moreover, the association of a single serotype b clonal lineage (designated the JP2 clone) and the aggressive form of periodontitis in adolescents has been investigated (266, 268). The JP2 clone of *A. actinomycetemcomitans* has a 530-bp deletion in the promoter region of the leukotoxin gene operon, which results in significant enhancement of leukotoxin production (269). The JP2 clone shows a limited geographical and ethnic host range, predominating in subjects of Northwestern African descent and apparently absent from populations of Northern European descent (270–272). Molecular analysis has indicated that the JP2 clone emerged as a distinct genotype approximately 2,400 years ago, in Mediterranean Africa (273). A 2-year prospective longitudinal cohort study of Moroccan adolescents who were initially free of periodontitis linked the JP2 clone of *A. actinomycetemcomitans* with a very substantial risk of development of periodontitis (266). In carriers with clones other than JP2, the risk was smaller but still attained statistical significance. Adolescent periodontitis is linked with non-JP2 strains in a widely different geographical and ethnic host range, but with a significantly lower relative risk than that observed for the JP2 clone (265, 270, 274–276).

Aggregatibacter aphrophilus and Brain Abscesses

A. aphrophilus is recovered frequently from supragingival plaque and saliva but constitutes only a small part of the subgingival microflora in health and periodontal disease (277). Infective endocarditis (see above) and cerebral abscesses are the most frequent invasive *A. aphrophilus* infections (278, 279), but epidural abscesses and intervertebral infections have also been reported (280–282).

Observations linking the bacterium with infections of the central nervous system anteceded the description of the species: strain ATCC 7901, isolated in 1932 from the spinal fluid of a child with a brain abscess, was deposited by Margaret Pittman as a representative of *H. parainfluenzae* (283) but was later identified as a V-factor-dependent isolate of *A. aphrophilus* (8, 284). In the 1960s, King and Tatum characterized invasive strains referred to the CDC during a 10-year period and noticed that 10 of 34 strains of *Haemophilus aphrophilus* (V-factor-independent isolates of *A. aphrophilus*) originated from brain abscesses, in marked contrast to none of 33 strains of *A. actinomycetemcomitans* (122). When bacteria from intracranial abscesses are identified to the species level, *A. aphrophilus* generally accounts for 2 to 7% of cultivable bacteria (285–288). Recent studies have attempted metagenomic analyses of bacterial species in brain abscesses by cloning and sequencing of PCR-amplified 16S rRNA genes (285, 289). One study cultured 36 bacteria from 30 of 51 specimens, including three isolates of *A. aphrophilus*, while 16S rRNA gene cloning and sequencing increased the number of patients with microbiologic detections to 39, including two additional patients with *A. aphrophilus* DNA; in total, 5 of 51 patients with brain abscess were positive for *A. aphrophilus* by culture and/or PCR (285). The frequent culture of *A. aphrophilus* from brain abscesses is disproportional to its presence among pharyngeal *Haemophilus* and *Aggregatibacter* species and among the total cultivable floras in saliva, subgingival crevices, and pharyngeal mucosa (277, 290). The species thus contains virulence properties enabling a specific association with intracranial abscess formation. The genome sequence of *A. aphrophilus* was recently published and encodes several putative virulence factors, including the products of a tight adherence cluster (*tad*) responsible for expression of long filamentous

fibrils, the products of a locus required for the assembly of type IVa pili, and a repertoire of adhesins that may participate in host colonization and pathogenesis (152).

Aggregatibacter segnis

A. segnis may be difficult or impossible to distinguish from *H. parainfluenzae* biotype V by phenotypic means. Although it is a well-known but rare cause of infective endocarditis (see above), the true prevalence of this bacterium in human infections is probably underreported. One study identified all isolates of *Haemophilus* cultured from blood from a single hospital by 16S rRNA gene sequencing and found 3 *A. segnis* isolates among 25 *Haemophilus* spp. cultured from 25 patients during a 7-year period (the others were 17 *H. influenzae*, 3 *A. aphrophilus*, and 2 *H. parainfluenzae* isolates) (247). Bacteremia with *H. parainfluenzae* and *Aggregatibacter* spp. affected adult patients with underlying diseases (247). Using a culture-independent molecular approach to analyze the microbiota of the oral cavity and the lungs of patients with ventilator-associated pneumonia, *A. segnis* was detected in the lung samples, a finding that has not been reported previously with culture-based methods (291).

CONCLUSIONS

With the disappearance of childhood *H. influenzae* serotype b meningitis in countries with effectual Hib vaccination, focus in the clinical microbiology laboratory has shifted somewhat, from the rapid detection of the bacterium from normally sterile body fluids to accurate identification of *H. influenzae* in specimens with concurrent growth of other bacteria. It has become clear that widely used methods are not always able to differentiate strains of *H. influenzae* reliably from *H. haemolyticus* and related organisms, and this has important implications for the clinical laboratory. Interpretation of the literature on respiratory tract colonization and infection by *H. influenzae* must be made with this important limitation in mind, as it can be expected that 15 to 20% of *H. influenzae* nasopharyngeal isolates from healthy volunteers will be identified erroneously by standard phenotypic tests. Due to the limited pathogenicity of *H. haemolyticus* and related organisms, the proportion of misidentified strains is significantly lower in clinical samples; however, a misidentification rate of 0.5 to 2% must be expected even among invasive strains.

A simple method to reliably distinguish *H. influenzae* from *H. haemolyticus* is not available. As there is evidence of recombinatorial transfer between the two species (292), which may even involve rRNA genes (153), no single gene can be expected to completely differentiate *H. influenzae* from its close relatives of minor pathogenic importance. The presence of *fucK* and conserved nucleotide motifs in *hpd* and *iga*, plus the absence of *sodC*, are typical for *H. influenzae*, while the opposite genotype is expected for *H. haemolyticus* and related organisms. These four traits can be determined by PCR on selected invasive isolates or for research purposes. However, some strains will exhibit a mixed genotype and must be identified finally by sequencing. A near-full-length 16S rRNA gene sequence or a multilocus sequence analysis of housekeeping genes is recommended.

In the near future, a large number of genomes from *H. influenzae*, *H. haemolyticus*, and related organisms will be available for analysis. Comparisons of core genomes and of total genomic contents will constitute a powerful data set for assessment of species boundaries. If an evolutionary continuum is revealed between *H. influenzae* and *H. haemolyticus*, a pragmatic approach to species delineation may be necessary. The insight gained from such studies will have great importance for the development of assays to distinguish *H. influenzae* from closely related species.

With the exception of *H. paraphrohaemolyticus*, the remaining four species of the *H. parainfluenzae* group and the three species of *Aggregatibacter* can be identified by phenotypic tests, although extensive characterization and expertise not readily available in most clinical laboratories are required. These species can also be identified accurately by 16S rRNA sequencing or by housekeeping gene sequencing using a multilocus approach. The delineation of *H. paraphrohaemolyticus* is not clear at present.

MALDI-TOF mass spectrometry is likely to have a profound effect on the workflow and results of the clinical microbiology laboratory. The technique is in an early stage, and identification algorithms and databases are continually being improved and refined. The limitations of mass spectrometry identification of *Haemophilus* and *Aggregatibacter* species are not known at present. Current identification algorithms recognize patterns of prominent proteins, which is adequate for the distinction of a very large number of bacterial species. Future improvements will probably incorporate algorithms making use of less prominent but taxonomically important peptide peaks. Whether mass spectrometry can be improved sufficiently to reliably distinguish *H. influenzae* from closely related species remains to be seen. It is clear that this method is capable of identifying many of the rare species of *Haemophilus* and *Aggregatibacter* at low cost and high speed. These species will therefore be identified more frequently from infections, which will increase our knowledge of their clinical significance.

The expansion and reorganization of the family *Pasteurellaceae* have been extensive. The family increased from 3 genera in 1995 to 18 genera in 2012, but most of these genera will not be found in clinical specimens unless they are incidentally introduced by animal bites or licks. Renaming of species originally defined by selected phenotypic traits has not necessarily come to an end. While the classification of species in the genus *Aggregatibacter* has reached a satisfying state by current standards, the phylogenetic position of *H. ducreyi* in the genus *Haemophilus* is not obvious, and this argument may even be extended to the *H. parainfluenzae* group (38). The ambition of a classification that reflects phylogeny can conflict with one of the principles of nomenclature, i.e., the stability of names. Some of these species are regularly encountered in clinical microbiology laboratories as causes of serious infections. A renaming may cause difficulties for health care workers, and misunderstandings can be dangerous. An eventual reclassification must be based on comprehensive analyses, and a conservative approach is presumably beneficial. The near future will see an overwhelming amount of genomic data that will require time to systematize, and even more time to interpret. If reclassification of familiar *Haemophilus* species at some time has to be considered, perhaps the methods of the clinical microbiology laboratory will then have evolved toward recognition of DNA sequences and protein patterns, and these methods, in contrast to selected phenotypic traits, are more inclined to match and benefit from a natural classification.

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